The Impact of Disease and Diet on the Metabolic Rate, Swimming Performance and Recovery of Atlantic Salmon (*Salmo salar* L.)

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Declaration

This thesis contains no material which has been accepted for a degree or diploma by the University or any other institution, except by way of background information and duly acknowledged in the thesis and to the best of my knowledge and belief no material previously published or written by another person except where due acknowledgements are made in the text of this thesis. Work within Chapter 5 was completed in conjunction with an experiment designed by Dr Matthew Miller who also wrote part of the methods and materials for the experiment. At the time of submission Chapter 2 has been published within peer reviewed journals (see appendix I).

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Abstract

Gill diseases represent one of the most abundant and economically important groups of diseases in fish, understanding the physiological response of fish to such diseases is crucial to the development of preventive measures. The majority of the research published on the physiological responses of the fish to changes in external stimuli, such as temperature, salinity or nutritional status have used apparently healthy fish. The study of the physiological responses of fish that are compromised with disease is inherently difficult however, due mainly to the invasive nature of the techniques used, which often result in mortality. Measuring the metabolic rate and swimming performance of a diseased organism is one of the least invasive techniques available for quantifying the impact of disease in fish Lethal sampling of the fish post-measurement can allow the researcher to determine possible reasons behind any change in metabolic performance. The nutritional status of the organism can also be a determining factor in the severity of the impact of disease. A reduction or a complete cessation of feeding can severely impact some physiological parameters that are associated with disease resistance, including a reduction in immune response, reduction in the organosomatic index of organs responsible for immune function, reduction in metabolic rate and a decrease in protein synthesis rates of some immunoregulatory organs, and therefore should always be taken into account when assessing the physiological impact of a disease. The composition of the diet can also play a significant role in physiological performance of an animal; research has suggested that the fatty acid profile of a feed can significantly affect the metabolic rate and swimming performance of Atlantic salmon. The aim of this thesis was to examine the physiological impact of two endemic gill diseases on the physiological performance of Atlantic salmon smolt, whilst simultaneously

examining the possible causes of any change in physiological performance. Furthermore, the effect of nutritional status on metabolic rate, by withholding feed and by altering the fatty acid profile of the feed, was also assessed.

Research was conducted to investigate the effect of *Tenacibaculum maritimum*, an acute necrotic bacterial gill infection, and amoebic gill disease (AGD), a proliferative gill disease, and feed deprivation on the metabolic rate of Atlantic salmon *Salmo salar*. For the *T. maritimum* infection, a significant decrease of 2.21 $\pm$ 0.97 $\mu M\ O_2\ g^{-1}\ h^{-1}$ in metabolic scope was found for the fed infected group, whilst the unfed infected group had a decrease of 3.16 $\pm$ 1.29 $\mu M\ O_2\ g^{-1}h^{-1}$, facilitated by a significant increase routine metabolic rate. Interestingly, all groups defended maximum metabolic rate despite the perceived loss of gill surface area. Increases in routine metabolic rates corresponded to a significant increase in blood plasma osmolality. A similar increase in routine metabolic rate (3.56 $\pm$ 0.62 $\mu M\ O_2\ g^{-1}\ h^{-1}$ for fed fish, 2.94 $\pm$ 0.55 $\mu M\ O_2\ g^{-1}\ h^{-1}$ for unfed fish) was observed for fish affected by AGD, despite the different modes of action between the two diseases, however fish affected by AGD defended metabolic scope. Further research was conducted in order to determine whether protein synthesis was one of the possible causes of the observed increase in routine metabolic rate of fish affected by AGD. Gill protein synthesis rates were examined using the flooding dose method, AGD affected fish had an mean synthesis rate of 13.56 $\pm$ 2.05 $\%.day^{-1}$, which was significantly ($P = 0.037$) higher than that of control fish (8.05 $\pm$ 0.97 $\%.\ day^{-1}$).. However the increase in protein synthesis is unlikely to be the sole reason for the observed increase in routine metabolic rate, instead it is likely that a number of physiological changes within the affected fish result in the observed increase in metabolic rate.

A further study was conducted to examine the effect of AGD on swimming performance. Salmon that had been inoculated via cohabitation with amoeba (*Neoparamoeba* spp) 14 days prior were given ramp critical swimming speed tests (U_{crit}) 45 min apart. Fish affected by AGD had significantly reduced recovery ratios (RR) (0.87 ± 0.02) compared to controls (1.00 ± 0.01), even at relatively low infection levels. A second experiment was performed examining the effects a freshwater bath on the repeat swimming performance of AGD affected salmon. Freshwater bathed fish showed a significant recovery in RR compared to non-bathed controls. The results indicated that AGD can significantly affect the swimming performance of Atlantic salmon, even at low infection levels.

A study was also conducted to determine the effect of replacing the fish oil component of a salmon feed with stearidonic acid (SDA) on the metabolic rate and recovery performance in Atlantic salmon. The results suggested that replacement of fish oil with SDA has little effect on metabolic rate when comparing the FA profile of the carcass of the fish and the observed metabolic rate and recovery. However, significant correlations were observed with the concentration of a number of fatty acids in the heart and the maximum metabolic rate of Atlantic salmon, suggesting that the FA profile of a diet can significantly affect the physiological performance of Atlantic salmon.

In combination the results have shown that gill diseases can have a significant effect on the physiological performance of Atlantic salmon. However, the nutritional status of the fish prior to infection has little impact on the severity of the impact of

the disease on metabolic rate. Furthermore, the research highlighted the link between the fatty acid profile of a diet and the physiological performance of the fish. Future studies in this area may benefit from the use of the methods outlined as a non-lethal means of quantifying the impact of disease on fish.

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List of Abbreviations

AGD	Amoebic gill disease
EPOC	Excess post exercise oxygen consumption
MO_2	Metabolic rate
$MO_{2\ rout}$	Routine metabolic rate
$MO_{2\ max}$	Maximum metabolic rate
RR	Recovery ratio
SMR	Standard metabolic rate
U_{crit}	Critical swimming speed
P_aO_2	Blood oxygen tension
PO_2	Blood oxygen saturation
PCO_2	Blood carbon dioxide saturation
C_s	RNA to protein ratio (mg RNA.g protein^{-1})
DO	Dissolved oxygen
ATP	Adenosine Triphosphate
FER	Feed efficiency ratio
k_{RNA}	RNA activity (k_s . g^{-1} RNA.day^{-1})
k_s	Fractional rate of protein synthesis (%.d^{-1})
[RNA]	Concentration of RNA (μg RNA. mg^{-1})
S_a	Free pool specific radioactivity of ^{3}H - phenylalanine (dpm.nmol^{-1})
S_b	Protein bound specific radioactivity of ^{3}H phenylalanine (dpm.nmol^1)
SGR	Specific growth rate (%. d^{-1})
WM	White muscle
SDA	Specific dynamic action
RR	Recovery ratio
SO	Stearidonic oil diet
CO	Canola oil diet
FO	Fish oil diet
SFA	Saturated fatty acids
MUFA,	Monounsaturated fatty acids
PUFA	Polyunsaturated fatty acids
DHA	Docosahexaenoic acid
DPA	Docosapentaenoic acid
EPA	Eicosapentaenoic acid
ETA	Eicosatetraenoic acid
SDA	Stearidonic acid
LA	Linoleic acid
ALA	α-Linolenic acid
LA	Linolenic acid
GLA	γ-Linolenic acid
AA	Arachidonic acid
TAG	Triacylglycerol
FFA	Free fatty acid
PL	Polar lipid

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Chapter 1 - General Introduction

1.1 INTRODUCTION

This thesis describes research on the metabolic impact of gill diseases in Atlantic salmon. Gill diseases make up a large proportion of reported disease cases in aquaculture, yet the impact they have on the physiology of the fish is poorly understood (Powell, 2007). Understanding the physiological impact of a disease on an organism is important as it allows for the development of handling procedures and therapeutic agents that may mitigate some of the deleterious effects of the disease. By quantifying the impact of disease on metabolism and physiological performance, an assessment can be made on the impact that disease has on specific life history traits, such as growth, reproduction and fitness. It also allows for an assessment to be made of the effect of disease pre-mortem, rather than relying on post-mortem sampling. Experiments described within this thesis aimed to determine the impact of two endemic gill diseases in Tasmania on the metabolic rate and physiological performance of Atlantic salmon. The aim of this chapter is to give a brief background to some of the terminology used in the study of metabolism, and some of the biotic and abiotic factors that can affect metabolism and swimming performance.

1.2 CATAGORIES OF METABOLISM

Metabolism can be categorised as standard, routine, active and maximum metabolic rate (Cech, 1990; Jobling, 1994). Standard metabolic rate (SMR) is the minimum

metabolic rate of animal, below which survival is impeded (Brett, 1964; Jobling, 1994). Routine metabolic rate ($MO_{2\,rout}$) is the metabolic rate of a quiescent fish, but not necessarily the lowest metabolic rate during a 24 h period (Fry, 1971). Active metabolic rate is the metabolic rate of a fish actively swimming at a sustainable level aerobically, whilst maximum metabolic rate ($MO_{2\,max}$) is the maximum metabolic rate that a fish can attain, this is usually achieved immediately after exhaustive exercise (Fry, 1947; Brett, 1964). Metabolic scope is usually determined by subtracting the standard metabolic rate from the maximum metabolic rate, although some authors use routine metabolic rate rather than standard metabolic rate due difficulties in obtaining an accurate measurement of standard metabolic rate (Cutts et al., 2002).

1.3 STANDARD AND ROUTINE METABOLIC RATE

By definition SMR is the metabolic rate of an animal free from muscular activity, producing a minimal amount of heat and is in a post-absorptive state (Brett and Groves, 1979; Priede, 1985; Jobling. 1994). The energy used when an organism is respiring at a minimal level is predominantly associated with the cost of homeostasis and maintaining bodily functions, including immune function, protein turnover, cellular integrity and osmoregulation (Brett, 1976; Houlihan et al., 1995; see section 1.4 of general introduction). Protein synthesis and osmoregulation account for a majority of the standard metabolic rate requirements in fish, protein synthesis alone can account for up to 20% of the maintenance energy expenditure, while osmoregulation can account for around 20-40% of total basal energy

requirements (Houlihan et al., 1988; Jobling, 1994; Houlihan et al., 1995; Carter and Houlihan, 2001).

However maintaining a fish in a respirometry chamber in a state in which is free from muscular activity in order to measure standard metabolic rate is difficult, especially for active fish such as salmon, with most measurements of standard metabolic rate relying on extrapolation from active metabolic rates of fish swum in a swim tunnel, or the continual monitoring of a fish in a respirometer over a 24 h period (Brett, 1964; Lucas and Priede, 1992; Jobling, 1994, see discussion below). Highly specialised equipment is required to take metabolic rate measurements of an actively swimming fish in a swim tunnel, therefore most researchers instead focus on taking routine metabolic rates ($MO_{2\ rout}$), which is the metabolic rate of a fish that is in a post-absorptive state and is exhibiting low levels of spontaneous activity (Brett, 1964; Brett, 1967; Jobling, 1994) Measurements of $MO_{2\ rout}$ are often taken as a way of quantifying the impact of a treatment on the energy expenditure of a fish, for example, temperature or salinity, or in the case of this thesis, disease, as a change in metabolic rate is indicative of a change in physiological status (see section 1.4, Table 1.1).

1.3.1 *Methods for measuring basal and routine metabolic rate*

There are a number of different methods for measuring SMR and $MO_{2\ rout}$ depending on the available equipment, the species of fish and the aim of the experiment.

1.3.1.1 Static respirometer

A static respirometer is a respirometer that, during the measurement of metabolic rate, has no water flowing through it (Cech, 1990). The respirometer box should ideally be air tight, although an air space at the top of the respirometer is acceptable if the time in which the measurement is being taken is small. To measure metabolic rate, water and air-flow to the respirometer are stopped, an initial oxygen concentration measurement is immediately taken, the fish is then left undisturbed for a set amount of time depending on the size of the fish relative to the respirometry chamber and the temperature of the water. A second final oxygen concentration measurement is taken, the drop in oxygen content in the respirometer and the weight of the fish are then used to calculate weight specific metabolic rate. Respirometers are either submerged or semi-submerged in water in order to minimise any changes in water temperature within the respirometer whilst there is no water flowing through the box. Advantages of this technique are that it requires a minimal amount of peripheral equipment, measurements can be made quickly and the technique allows for the metabolic rates of a number of fish to be measured simultaneously. Furthermore, as the measurement can be made quickly, further physiological assessments can be made within a short timeframe, for example the fish can be placed in a swim tunnel to measure U_{crit} (Wilson et al., 2007) or chased to exhaustion to measure $MO_{2\ max}$ and excess post-exercise oxygen consumption (EPOC) (Lee et al., 2003). There are limitations to this method; the activity of the fish within the respirometer cannot often be accounted for if the boxes do not allow for direct observation of the fish (Cech, 1990). Also, the metabolic rate of a fish fluctuates throughout the day, so a measurement of metabolic rate at a single time point is unlikely to be at a point at which the fish is respiring at a minimal level,

hence this method is only suitable for measuring routine metabolic rate. Several studies have utilised this method, including work examining the effect of disease and diet composition on the routine metabolic rate of fish (Table 1.1). Of particular interest to this thesis, Powell et al. (2005) demonstrated that an increase in $MO_{2\ rout}$ could be detected in fish affected by *Loma salmonae*, and Wilson et al (2007) deemed the method suitable for attempting to detect differences in $MO_{2\ rout}$ in fish fed diets with various levels of fish oil replacement.

1.3.1.2 *Flow through respirometers*

Flow-through respirometers are often designed similarly to static respirometers, the main difference being that oxygen measurements are taken from the incoming and outgoing water streams from the respirometer. The water is allowed to flow continuously through the respirometer at a rate that allows a detectable drop in oxygen concentration from the incoming and outgoing water supply. The advantage to this system is that it allows for continuous measurement of $MO_{2\ rout}$ without a constant interruption of water flow that is required in static measurements. Standard metabolic rate can also be determined if the measurement period is suitably long, ideally longer than 8 h (McKenzie, 2001). Many studies have successfully used this method to measure metabolic rate continuously over a 24 h period or longer (Table 1.1), however, measuring metabolic rates of fish over an extended period of time whilst the fish is confined within a respirometer may pose inherent risks to the fishes health, particularly if the fish is stressed or sick prior to measurement. If an active fish such as salmon becomes startled or stressed whilst confined, there is a risk that the fish may damage itself on the walls of the respirometer, the risk increases the longer the fish is confined. This is particularly important in disease

studies where a fish may be physiologically compromised or stressed prior to any measurements; any further damage or stress could potentially lead to mortality (Powell, 2007). Therefore this method may be more suited to measurements of fish that are in ideal health in order to minimise the risk of mortality.

1.3.1.3 *Brett type respirometers*

Another method for determining basal or standard metabolic rate is to place a fish into a swim tunnel and increase the water velocity in step-wise increments. Oxygen consumption rates are measured with each increase in velocity, standard metabolic rate is then calculated by extrapolating back to zero activity (Brett, 1964; Brett and Glass, 1973; Brett and Groves, 1979). However there are difficulties in this assumption, chiefly there are physiological differences between an actively swimming fish and a quiescent fish (Cech, 1990). Actively swimming fish release epinephrine into the blood stream, which dilates the blood vessels in the gill and changes the haemoglobin binding affinity for oxygen (Wood and Shelton, 1980; Nikinmaa, 1982). Furthermore, fish swimming at higher velocities may engage anaerobic metabolism, making extrapolation back to basal metabolic rate problematic (Cech, 1990).

Table 1.1.

Comparisons of different methods used in various selected studies to measure routine and standard metabolic rate.

Species	MO_2	Method	Measurement frequency	Experiment
Atlantic salmon[1]	routine	flow through	3 measurements over 3 hr	Feed deprivation
Atlantic salmon[2]	routine	flow through	every 4hr over 24hr	Feed deprivation
Sockeye salmon[3]	standard	extrapolation	1 measurement	Size and temperature
Atlantic cod[4]	routine	flow through	single measurement	Feeding and protein synthesis
Atlantic salmon[5]	routine	flow through	every 3 hrs over 24 hr	Parr/smolt comparison
Atlantic salmon[6]	standard	flow through	every 10min over 48hr	Parr/smolt comparison
Turbot[7]	standard	static	every 90 min over 24 hr	Temperature and DO
Sockeye salmon[8]	standard	extrapolation	1 measurement	Fundamentals
Rainbow trout[9]	routine	static	every hr over 4 days	Density

Atlantic salmon[10]	standard	flow through	single measurement	Metabolic scope
Zebrafish[11]	standard	static/extrapolation	single measurement	Group-individual MO_2
Adriatic sturgeon[12]	standard	extrapolation	single measurement	Change in salinity
Coho salmon[13]	standard	extrapolation	single measurement	Change in salinity
Sea bass[14]	routine	static	single measurement	Change in salinity
Sockeye salmon[15]	routine	static	single measurement	Temperature
Sockeye salmon[16]	routine	static	single measurement	Disease
Rainbow trout[17]	standard	extrapolation	single measurement	Disease
Rainbow trout[18]	routine	static	single measurement	Disease
Sockeye salmon[19]	routine	?	Two measurements in 1 hr	Disease
Pacific salmon[20]	routine	static	single measurement	Field measurements
Eel[21]	routine	static	single measurement	Migration costs
European eel[22]	routine	static	every 10 min for 8 hr	Dietary FA content

Adriatic sturgeon[23]	routine	static	every 10 min for 8 hr	Dietary FA content
Atlantic salmon[24]	routine	static	single measurement	Dietary FA content
Seabass[25]	standard	extrapolation	single measurement	Dietary FA content
Green sturgeon[26]	routine	static	single measurement	Stress

1. O'Connor et al 2002
2. Cook et al., 2000
3. Brett and Glass, 1973
4. Lyndon et al., 1992
5. Higgins 1985
6. Maxime et al., 1989
7. Mallekh and Lagardére, 200
8. Brett 1964
9. Lefrancois et al., 2001
10. Cutts et al., 2002
11. Lucas and Priede, 1992
12. McKenzie et al., 2001
13. Morgan and Iwama, 1998
14. Dalla Via et al., 1998
15. Lee et al., 2003
16. Tierney and Farrell, 2004
17. Kumaraguru et al., 1995
18. Powell et al., 2005
19. Wagner et al., 2005
20. Farrell et al., 2003
21. Van Ginneken et al., 2005
22. McKenzie et al., 2000
23. McKenzie et al., 1997
24. Wilson et al., 2007
25. Chatelier et al., 2006
26. Lankford et al., 2005

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1.4 SWIMMING PERFORMANCE AND MAXIMUM METABOLIC RATE

Swimming performance tests are a useful method for studying the regulatory processes associated with physiological fitness and swimming capacity, because they allow the rate limiting factors to be determined in exercise performance and recovery in fish (Brett, 1964; Kieffer et al., 1994; Burgetz et al., 1998). Factors affecting fish health and performance can be assessed in a non-lethal manner, studies have successfully assessed the impact of changes in water temperature (Brett and Glass, 1973; Dickson and Kramer, 1971; Schurmann and Steffensen, 1997; Lee et al., 2003), pollution (Webb and Brett, 1973), hypoxia (Farrell et al., 1998), ploidy (Altimiras et al., 2002), stress (Gregory and Wood, 1999) as well as disease (Tierney and Farrell, 2004; Jain et al., 1998). In most cases a swimming performance test assesses the ability of fish to swim over a prolonged period of time, and can range from 20 s to 200 min, over which the fishes aerobic and anaerobic capacity is assessed (Beamish, 1978; Jobling, 1994). The most typical form of swimming test used is the critical swimming speed test (U_{crit}), in which fish are subjected to step-wise increases in water velocity over a defined period of time until fatigued (Brett, 1964). A second swimming performance test, known as a repeat swimming performance test can be added, in order to assess the ability of fish to recover from exhaustive exercise (Farrell et al., 1998; 2003; Jain et al., 1998; Tierney and Farrell, 2004). Critical swimming speed tests are based on prolonged exercise in which the predominantly aerobic red muscle tissue is used, however at approximately 70-

80% U_{crit} most fish will switch to use the predominantly anaerobic white muscle tissue in order to hold station in the water column (Burgetz et al., 1998). Alternatively, some researchers use burst-type swimming tests in which fish are kept in a constant state of acceleration, fish swum in this manner will exhaust quickly because they predominantly use anaerobic metabolism to provide energy for locomotion (Reidy et al., 1995; Cutts et al., 2002; Powell et al., 2005). The metabolic rate of a fish immediately after this form of swimming test is referred to as maximum metabolic rate (Reidy et al., 1995; Cutts et al., 2002). Swimming performance tests that use this method of exhaustion elicit a metabolic rate post exhaustion that is significantly higher than that obtained by swimming a fish in a swim tunnel (Reidy et al., 1995). Whilst anaerobic metabolism is inefficient at yielding energy when compared to that of aerobic metabolism, it provides much needed energy during short periods of strenuous exercise, indeed the anaerobic capacity of a fish can often be the critical factor in the short-term survival of fish in stressful life threatening conditions (Kieffer et al., 1996; Burgetz et al., 1998).

There are a number of biochemical processes that differentiate anaerobic from aerobic metabolism, primarily, there is a rapid exhaustion of ATP, creatine phosphate and glycogen reserves, with rapid accumulation of lactate in white muscle tissue (Moyes et al., 1993; Burgetz et al., 1998). Immediately following exhaustive exercise is a sustained increase in oxygen consumption, the difference between the oxygen consumption rate at this point in time and the routine or standard metabolic rate is known as the metabolic scope, and indicates the maximum amount of oxygen available for fish at a particular

temperature. It reflects the energy costs of all types of activity over and above that required for standard metabolism (Fry, 1947). The total oxygen consumed after activity until the metabolic rate returns to standard levels is known as excess post-exercise oxygen consumption (EPOC), a measure often used to assess the ability of fish to recovery after burst type swimming activity, although it a useful measure for assessing recovery after U_{crit} tests (Brett, 1964; Gaesser and Brooks, 1984; Reidy et al., 1995; Scarabello et al., 1991; Scarabello et al., 1992; Lee et al., 2003). The sustained increase in oxygen consumption rates above standard and routine levels is due to the cost of re-oxidising lactate to glycogen for fuel, the recovery of ATP and creatine phosphate, replenishing internal oxygen stores and the re-establishment of acid-base and ionic balances and fluid volume (Jobling, 1994; Reidy et al., 1995; Lee et al., 2003). The rate of recovery is highly variable and can range from several minutes to several hours, depending on species, intensity of exercise, temperature and the condition of the fish (Reidy et al., 1995; Lee et al., 2003).

1.5 INFLUENCES ON METABOLIC RATE AND SWIMMING PERFORMANCE

The metabolic rate and swimming performance of fish are sensitive to a number of biotic (weight, condition factor, growth rate) and abiotic (temperature, salinity, water hardness) factors. The effect of these factors on metabolic rate and swimming performance are well documented (see reviews Brett and Groves, 1979; Jobling, 1994), the following section discusses the factors that are most relevant to this thesis.

1.5.1 *Gill diseases*

Gill diseases are likely to have a significant effect on the respiratory physiology of fish, as the gill plays a major role in regulating metabolic rate and ion regulation. The gill is covered in a thin epithelium (discussed below) that has a large surface area that allows the diffusional transfer of respiratory gases in and out of the blood down a partial pressure gradient (See review by Perry and MacDonald, 1993; Gilmour, 1998; Powell, 2007). Rates of oxygen consumption are limited by the functional surface area of the gill, the blood-water diffusional distance and the partial pressure gradients of O_2, maintained by the afferent and efferent gas partial pressures of blood in the gill. Changes to these parameter will ultimately affect MO_2 (Perry and MacDonald, 1993; Gilmour, 1998; Powell, 2007). Changes in blood flow will also affect MO_2, possibly more so in some cases than structural changes, as MO_2 is intrinsically linked to cardiac output (Powell, 2007). Most gill diseases, both proliferative and necrotic, affect at least one, but often a majority of the aforementioned parameters, thus the potential to severely affect respiration is much more pronounced in diseases of the gill.

There are two main types of gill diseases, those that are necrotic which result in an erosion of the gill epithelium, and those that are proliferative which result in a hyperplastic response from the epithelial cells (mucous cells, pavement cells, chloride cells) (Powell, 2007). Necrotic diseases are associated with extensive necrosis to the respiratory epithelium, often resulting in the progressive erosion

of the lamellae and filaments (Powell, 2007). Experiments with *Tenacibaculum maritimum*, an aggressive necrotising infection in marine fish, showed that extensive erosion of lamellae and gill filaments had little effect on blood gas levels and the saturation of haemoglobin (Powell et al., 2005a). Evidence suggested that mortality was caused by an osmoregulatory dysfunction rather than respiratory failure (Powell et al., 2004; 2005a). An osmoregulatory dysfunction, which would result in an increase in plasma osmolality for fish in saltwater, can be expected to cause a significant increase in metabolic rate. It has been estimated that $Na^{+}.K^{+}$ -ATPase activity, which is responsible for maintaining cellular homeostasis and ion transport, accounts for 20-40% of standard energy expenditure, therefore any change in the osmoregulatory capabilities of a fish will presumably affect $MO_{2\ rout}$.

Epithelial cell proliferation is a common inflammatory response to bacterial and parasitic pathogens (Byrne et al., 1991; 1995; Adams and Nowak, 2003; Powell and Nowak, 2003; Powell, 2007). Whilst proliferative diseases effectively reduce the available surface area for respiration by increasing the blood-water diffusion barrier, oxygen diffusion rates are generally not affected as the proliferation is not uniform, thus leaving large areas of gill epithelium unaffected (Powell et al., 2000; Powell and Nowak, 2003; Powell, 2007). Blood oxygen saturation (PO_2) values of resting Atlantic salmon with AGD were only slightly lower than that of controls, similar results were found for brook trout and rainbow trout affected by bacterial gill disease (*Flavobacterium branchiophilum*) (Byrne et al., 1991; 1995; Powell et al., 2000; Powell and Nowak, 2003). It is important to note that PO_2 values were taken in fish that

were quiescent, no research on the effect of gill diseases on blood PO_2 levels during strenuous exercise has been performed, this would be of interest as fish during periods of high intensity exercise require more available gill surface area for respiration, thus potentially mitigating any compensatory measures the fish has in place in order to cope with the effects of disease.

1.5.2 Protein synthesis

One of the characteristic features of proliferative diseases is an undifferentiated proliferation of epithelial cells facilitated by an increase in protein synthesis. Protein synthesis can account for between 11 – 24% of the energetic cost associated with routine metabolism, in fast growing juvenile fish it can account for up to 42% of the total energetic requirements (Carter and Houlihan, 2001). The minimum energetic cost of protein synthesis is assumed to be 40 mmol ATP per g of protein synthesised plus 10 ATP per g of protein transported (Houlihan et al., 1988, Carter et al., 2001). The high energetic requirements are mainly associated with the process of mRNA translation, which can be correlated to oxygen consumption rates (Houlihan, 1991).

There are a number of biotic and abiotic factors that can modify the protein synthesis rates of the whole body or specific organs of the fish (see reviews Carter and Houlihan, 2001; Fraser and Rogers, 2007). The majority of the literature on protein synthesis examines the effect of temperature on protein synthesis rates, with protein synthesis rates generally increasing with increasing temperature (see reviews McCarthy and Houlihan, 1997; Carter and Houlihan,

2001; Fraser and Rogers, 2007). Other environmental factors such as salinity and oxygen levels can also have an affect on protein synthesis rates. Environmental pollution significantly affects protein synthesis, particularly gill protein synthesis since the gill has the highest surface area of all tissues exposed to the environment (Lyndon and Houlihan, 1998). For example, gill protein synthesis rates of rainbow trout gills exposed to a sublethal concentration of aluminium in an acidic environment significantly increased immediately following exposure, returning to normal levels after 15 days, suggesting that increased synthesis rates were associated with an increase in repair of gill tissue (Wilson et al., 1996). Importantly, no work to date has been performed examining the effect of disease on protein synthesis rates in fish. Increases in protein synthesis rates associated with disease in humans have been shown to account for approximately 50% of the observed increase in routine metabolic rate, increases in protein synthesis occurred due to an increase in the synthesis of acute phase inflammatory proteins and antibodies (Borel et al., 1998). Also of particular interest is whether any observed changes in protein synthesis in the gills associated with gill diseases are accompanied by a concomitant change in other organs, particularly white muscle tissue as white muscle tissue synthesis rates are indicative of whole body synthesis rates (Carter and Houlihan, 2001). An often observe phenomena in diseased animals is a period of anorexia (see general introduction section 1.5.3 and general discussion). Decreases in feed intake are associated with a decrease in whole body synthesis rates, therefore any increase in gill protein synthesis rates associated with disease may be proportionally higher due to a decrease in whole body synthesis rates (Carter and Houlihan, 2001).

There are a number of different methods available for measuring protein synthesis rates. The main methods used in fish physiology studies are the constant infusion method (Haschemeyer et al., 1979), the flooding dose method (Garlick et al., 1980), and end point analysis (Carter et al., 1994). The flooding dose requires a large dose of a radioactive labelled amino acid (^{3}H phenylalanine) to be injected into the fish in order to flood the amino acid free pools, incorporation occurs at all levels from cellular to organismal (reviewed Garlick et al., 1994; Houlihan et al., 1995; Carter and Houlihan, 2001). The bound ^{3}H phenylalanine and the ^{3}H phenylalanine in the free pool are then analysed in order to determine protein synthesis rates. The use of this method is based on a number of assumptions, primarily that the flooding dose of radiolabelled ^{3}H-phenylalaine does not affect the rates of protein synthesis (Houlihan et al., 1995, Carter and Houlihan, 2001). Furthermore, it is important that the specific radioactivity in the free pool must be elevated and remain stable over the the incorportation period, and the incorporation of the radiolabel into the protein is linear over time (Houlihan et al., 1995). Constant infusion involves administering a radioactive tracer over an extended period of time allowing assessments to be made of protein synthesis rates over a long term period, however it is rarely used in fish due to the difficulty in attaching a suitable administrating device to the body of a live fish (Haschemeyer and Smith, 1979; Fauconneau and Tesseraud, 1990). End point analysis is used to estimate whole body synthesis rates by administering a stable isotope, usually ^{15}N, and examining the concentrations of the stable isotope in the end product of protein metabolism (ammonia) (Carter et al., 1994). "This method is particularly

advantageous when multiple measurements of protein synthesis over a period of days is required, for example, when examining the effect of diel fluctuations in protein synthesis rates (Carter et al., 1994; Houlihan et al., 1995)"

For this particular study the flooding dose method was chosen because it can be used to analyse short-term protein synthesis rates and allows tissues to be investigated, this allowed for the fractional synthesis rates of gill tissue in response to disease to be specifically assessed against control levels.

1.5.3 Diet

Feeding can increase routine metabolic rates 2 – 3 times that of resting metabolic rate levels and are usually elevated for several hours post feeding depending on ration, composition and temperature. This is variously termed specific dynamic action (SDA), heat increment (HI) and feeding respiration (Rf) (Jobling, 1994; O'Connor et al., 2000; Cutts et al., 2002). Specific dynamic action is partly due to the metabolic demands associated with the actual act of feeding, but is mainly associated with the cost of digestion and assimilation, deamination of amino acids, production of excretory products and biosynthesis, turnover and deposition of tissue (Carter and Brafield, 1991; 1992; Houlihan et al., 1995). Likewise, fish that have been starved for an extended period of time significantly reduce their routine metabolic rates (O'Connor et al., 2000; Cook et al., 2000). A reduction in metabolic rate associated with prolonged periods of starvation is thought to have evolved in fish as a way of reducing their energetic needs during times of famine, this is particularly relevant for salmonid species

that undergo extensive migrations from feeding grounds to spawning grounds often without feeding (Cook et al., 2000). The reduction in metabolic rate is facilitated by a decrease in the relative size of organs, a decrease in protein turnover and a distinct change in the biochemical composition of the body tissues (Cook et al., 2000; O'Connor et al., 2000).

Often disease outbreaks are associated with a period of anorexia, whilst regarded as an undesirable manifestation of the disease, some evidence suggests that this may in fact be part of the hosts natural defence mechanisms (Pirhonen et al., 2003). One theory is that anorexia promotes an effective immune response, another being that starvation allows the animal to be more selective in its source of food, potentially minimising the risk of further infection (Kyriazakis et al., 1998). In mice that have been starved for a prolonged period there is a significant increase in the number of macrophages and natural killer cell activity (Wing and Young, 1980; Wing et al., 1983; Wing et al., 1986). Atlantic salmon that had been starved prior to infection with *Vibrio salmonicida* showed a significant increase in survival rates (Damsgård et al., 1998), similar results were found for channel catfish *Ictalurus punctatus* during an *Edwardsiella ictaluri* epzootic (Wise and Johnson, 1998) and rainbow trout exposed to *Flavobacterium branchiophilum* (MacPhee et al., 1995). One explanation for a reduction in mortality is a reduction in the interaction between the host and the pathogen. Bacteria such as *Aeromonas salmonicida* are hydrophobic in nature, pellets that are being fed to fish could be coated in the pathogen as it passes through the water surface prior to being eaten (Wise and Johnson, 1998). Another explanation that is particularly pertinent to gill

diseases, is that feeding induces physiological changes in the host that facilitate the proliferation of disease in the host (MacPhee et al., 1995). Rainbow trout that were not fed after being exposed to *Flavobacterium branchiophilum,* the aetiological agent of bacterial gill disease (BGD), had significantly reduced mortality rates compared to fed controls (MacPhee et al., 1995). The authors suggested that this was caused by a reduction in the excretory by-products of feeding, chiefly ammonia, from the gills (MacPhee et al., 1995). Excretion of ammonia and other by-products, including carbon dioxide, can create a micro-environment surrounding the gill epithelium that is conducive to bacterial growth by providing a nutrient rich zone in which the bacteria can proliferate (MacPhee et al., 1995). Whether this holds true for all gill diseases is unknown as different pathogens have different mechanisms for attachment to the host and are stimulated by different environmental conditions. Control measures for disease that do not involve the use of chemotherapeutics in the livestock industry is highly desired, thus research into the effect of withholding feed on disease proliferation is of particular importance.

1.5.3.1 Dietary composition

Research into the physiological effects of diet and nutrition has begun to diversify away from the effects of ration, toward the effects of specific dietary components. Upward trends in fish oil prices has lead to a field of research examining the physiological effects of fish oil replacements, particularly in salmon feeds. Whilst complete replacement is the ultimate goal, this has proved rather difficult as salmonids have a requirement for eicosapentaenoic acid

(EPA; 20:5ω-3) and docosahexaenoic acid (DHA; 22: 6ω3) at around 10% of the total lipids in the diet, with fish oil being the major source of EPA and DHA (Higgs and Dong, 2000). Marine fish oil in salmonids can be replaced with 50 – 80% of a lipid sourced from terrestrial plant or animal sources without any effect on growth or growth efficiency (Dosanjh et al., 1988; Thomassen and Røsjø, 1989; Jordel et al., 2007). However there is some, all be it limited, evidence to suggest that there are severe physiological repercussions when replacing fish oil with terrestrial based oil sources, such as cardiac lesions and a higher susceptibility to stress induced mortality, especially if the fatty acid profile of the oil is not meeting the requirements of the species (McKenzie et al., 2000; McKenzie et al., 2001).

The research to date on the physiological effects of fish oil replacement has shown that no one particular physiological outcome can be related to a deficiency or an excess of any one particular fatty acid, instead it suggests that there is an interaction between specific fatty acids that may be affecting metabolic rate and swimming performance (Wagner et al., 2004). Two studies, McKenzie et al (1998) and Chatelier et al (2006) found an inverse relationship between α-linolenic acid (ALA; 18:3ω3), EPA and DHA and U_{crit} but a direct relationship between 18 carbon PUFA's and U_{crit}. However, conflicting results showed U_{crit} decreased with increasing supplementation of anchovy oil (high in DHA and EPA) with poultry oil, but not with flaxseed or sunflower oils (Wagner et al., 2004). However no effect on metabolic rate or swimming performance was observed when replacing anchovy oil with poultry fat, canola oil or flaxseed oil (Wilson et al., 2007). The results suggest that dietary oil

source can have a significant effect on a number of physiological variables, and as such any oil replacement research would be well advised to determine the effect of the oil replacement on the physiology of the fish in question before commercial scale replacement programs are enacted. Studies into the effect of oil replacement on metabolic rate and swimming performance should be included in a range of measurements to provide a wider range of responses with which to ensure fish oil replacement was unlikely to affect the physiological performance and long term health of the animal. One such fish oil replacement that is being investigated for Atlantic salmon is the use of stearidonic acid (SDA; 18:4n3) in the form of oil extracted from the plant *Echium plantagineum,* as trials have suggested that salmon parr are capable of using SDA as a precursor for EPA (Miller et al., 2007). However it is unknown whether salmon smolt can convert SDA through to EPA, and if indeed this is the case, whether the physiological consequences associated with a reduction of EPA in the diet observed in previous studies remain true.

1.6 Respiratory Physiology

1.6.1 Gill function and structure

The teleost fish gill is a multi-purpose organ that is made up of four pairs of gill arches from which long narrow filaments, known as primary lamellae, project laterally (Evan et al., 2005). From each primary lamella numerous evenly spaced plate like secondary lamellae project at right angles along the length of the primary lamellae. The secondary lamellae provide the majority of the

respiratory surface area for gaseous exchange, and are comprised of two separate epithelial sheets held apart by a series of pillar cells (Evans et al., 2005). Blood flow within the lamellae operates in a counter current manner to that of the surrounding environment in order to maximise gaseous exchange, and the transfer of gases between the outside environment and the extracellular fluid relies primarily on passive diffusion (Randall, 1990). Oxygen uptake is perfusion limited rather than diffusion limited due to the rapid equilibration between the blood and the inspired water (Perry and Gilmore, 1993). A thin layer of cells known as the epithelium covers the gill filaments and lamellae, and forms a boundary layer between the extracellular fluids of the fish and the external environment. The epithelium comprises of a number of different cell types differentiated primarily by function and include mucous cells, chloride cells and pavement cells, the later makes up >90% of the total gill surface area and plays a prominent role in gaseous exchange (Powell, 2007). In order to maximise gas exchange, the epithelial cell layer is rarely more than a few cell layer thick, therefore any changes to epithelial thickness, for example through hyperplasia, can potentially affect gaseous exchange by increasing the diffusional distance between the extracellular fluid and the outside environment (Powell, 2007). However gaseous exchange rates are not static, rather, a number of variables can be adjusted within the gill in order to maximise gaseous exchange. For one, the functional gill surface area can be increased. Under resting conditions the gill is not equally perfused nor fully ventilated, which essentially means there is a difference between the total surface area, estimated from morphometric studies, and functional gill surface area, which is the surface area available for gaseous exchange (Powell, 2007). This means that there is

substantial scope for recruitment of under-perfused lamellae via redistribution of blood flow within the lamellae (Booth, 1979; Olson, 1979).

1.7 PATHOPHYSIOLOICAL EFFECTS OF DISEASE

1.7.1 Amoebic Gill Disease (AGD)

It was initially thought that the mortality of fish with AGD was due to respiratory failure, fish affected by AGD show obvious signs of oxygen deprivation such as lethargy and respiratory distress, and an increase in amplitude and rate of operculum movements (Kent et al., 1988; Munday et al., 1990; Roger and McArdle, 1996; Fisk et al., 2002). Recent studies have now shown that fish affected by AGD do not suffer from significant hypoxemia. Powell et al (2000) showed that while there is a slight decreased in P_aO_2 levels in AGD affected fish due to a restriction in gas transfer under normoxic conditions, under hypoxic conditions physiological mechanisms such as a redistribution of blood flow and lamellae recruitment are able to compensate for the restriction, thus maintaining respiration. However, there are significant acid base disturbances associated with AGD due to an increase in PCO_2 levels within the blood (Powell et al., 2000). Increased PCO_2 levels were associated with a lowering of the blood pH under normoxic and hypoxic conditions, thought to have been brought on by a reduction in gill surface area and an increase in mucous secretion. AGD also has profound cardiovascular effects on Atlantic salmon. AGD affected salmon have significantly elevated dorsal aortic blood pressure and elevated system vascular resistance with an associated cardiac

dysfunction (Powell et al., 2002a). Recent work by Leef et al (2005) has also shown a significant drop in cardiac output in fish affected by AGD even at relatively low infection levels. Chronic AGD has also been shown to affect cardiac morphology. Increased ventricle length and thickening of the ventricular compact muscle are thought to be compensatory measures to cope with the increased aortic pressure associated with AGD (Powell et al., 2002b). Any changes in cardiac morphology can have a profound impact on the cardiac function as the two are intrinsically linked (Graham and Farrell, 1992; Franklin and Axelsson, 1994; Tota and Gattuso, 1996). The change in ventricular shape observed in fish with chronic AGD is expected to result in a reduction in contractile force, and the increase in myocardium tissue thickness may be a compensatory measure resulting in a change in stroke volume to offset the high arterial afterload. As cardiac performance is intrinsically linked to physical performance parameters such as swimming performance, it is thought that the aforementioned cardiovascular effects will likely have a significant impact on the ability of fish to maintain physical performance equivalent to that of an unaffected fish.

1.7.2 *Tenacibaculum maritimum*

Tenacibaculum maritimum is a fish pathogen that causes significant mortalities in the commercial production of many fish species globally. The bacteria was first isolated from intensively cultured sea bream in Japan (Masumura and Wakabayashi, 1977) and was later reported in Japanese flounder *Paralichthus olivaceus*, leather jacket *Aluterus monoceros*, rock bream *Oplegnathus fasciatus* and plaice *Cleisthenes pinetorum herzenstenini* as well as several other fish

species (Baxa et al., 1986; Wakabayashi et al., 1986). Since then it has become a significant pathogen in a number of other cultured species worldwide. In Australia the main species affected are Atlantic salmon and rainbow trout, the disease usually manifests as ulcerated skin lesions and mortalities of up to 30% were reported in some farms (Handlinger et al., 1997). The disease usually progresses from a distinct scale loss, through to the erosion of the dermis, resulting in the exposure of the underlying musculature of the fish. Lesions are often present on the gill tissue as well, and appear as yellow to grey patches with an obvious erosion of the gill structure. It is thought that toxins associated with *T. maritimum* play a major role in the pathogenicity of *T. maritimum*, a distinct lack of inflammatory cells in histological samples of Atlantic salmon challenged with *T. maritimum* give some evidence to this theory (Soltani, 1995). Infection with *T. maritimum* directly onto the gill tissue doesn't appear to affect the blood P_aO_2 or the C_aO_2 of Atlantic salmon (Powell et al., 2005). This is despite the gills of infected fish showing significant areas of branchial necrosis and erosion, and it may be concluded that mortality is most likely caused by an ion regulatory failure rather than a respiratory failure. Powell et al (2004) did find a significant increase in plasma lactate levels pre-mortem, suggesting some form of respiratory failure, however the authors could not determine whether this was due to a diffusive or perfusive limitations to gas exchange. It appears that the branchial necrosis causes a strong osmoregulatory disturbance in Atlantic salmon, with a significant increase in blood plasma osmolality (Powell et al., 2004).

1.8 Thesis Aims

The fundamental aim of this thesis was to investigate the effect of gill diseases on the metabolic rate and swimming performance of Atlantic salmon. Furthermore, previous research on bacteria gill disease demonstrated that a reduction in ration effectively reduced mortality post infection, therefore the interaction between with holding feed and disease was also examined (MacPhee et al., 1995). In addition, the effect of a fish oil replacement diet on the metabolic rate on Atlantic salmon is also described.

Chapter 2 – The effect of *Tenacibaculum maritimum* and feed deprivation on the metabolic rate of Atlantic salmon (Published in Diseases of Aquatic Organisms, 78: 29-36, 2007)

A distinct ion regulatory disturbance is associated with infection with *T. maritimum* (Powell et al., 2004; 2005). This is due to the necrosis of the dermis of the skin, or in the case of gill tissue, the erosion of the epithelium layer. Any ion regulatory disturbance must, in theory at least, be met with an increase in metabolic rate as the enzymes responsible for maintaining ion regulatory homeostasis work to correct any osmotic imbalances brought on by the disease (Morgan and Iwama, 1998). Given that Na^+K^+-ATPase activity can account for up to 40% of standard metabolic rate, any change in activity should be detectable when measuring oxygen consumption rates (Jobling, 1994; Morgan and Iwama, 1998). Indeed many studies have shown that an increase in salinity, which is analogous to the ion respiratory disturbances seen in fish with necrotic lesions in a saltwater environment, significantly increases metabolic rate and

decreases metabolic scope (Morgan and Iwama, 1991; Dalla Via et al., 1998; McKenzie et al., 2001; Chatelier et al., 2005). This research aims to determine the extent to which routine and maximum metabolic rate, and consequently metabolic scope, is affected by the ion regulatory disturbances brought on by a necrotizing gill disease. Furthermore, withholding feed has been shown to be a successful strategy for disease management for some diseases (Shoemaker et al. 2003). Feed deprived Atlantic salmon had a 2% cumulative mortality compared to 36% mortality in fed animals when experimentally challenged with a pathogenic bacteria *Vibrio salmonicida* (Damsgard et al., 1998). Withholding feed also has a significant effect on metabolic rate, therefore the effect of withholding feed on metabolic rate and disease was also examined for both the *T. maritimum* and AGD metabolic rate experiments.

Chapter 3 the effect of amoebic gill disease and feed deprivation on the metabolic rate of Atlantic salmon

Previous studies have shown that AGD is associated with pronounced acid-base disturbances due to extensive branchial mucus secretion, which impairs CO_2 excretion without limiting O_2 uptake (Powell et al., 2000). A significant reduction in cardiac output has also been associated with AGD, with cardiac performance intrinsically linked to metabolic performance (Leef et al., 2005). A reduction in gill surface area, such as that caused by AGD lesions, have been shown to result in a reduction in active metabolic rate (Duthie and Hughes, 1987; Davidson et al., 1990). Furthermore, a reduction in gill surface area has implication for ventilation, osmoregulation and recovery from exhaustive

exercise (Duthie and Hughes, 1987; Kaufmann, 1990). Given what is known about the pathophysiology of fish affected by AGD, in this study it was hypothesised that as AGD progressed in terms of number affected filaments, routine metabolic rate would increase with a concurrent decrease in maximum metabolic rate, ultimately affecting metabolic scope.

Chapter 4. The effect of AGD on the swimming performance and recovery in Atlantic salmon

Amoebic gill disease has shown to cause a significant cardio-respiratory dysfunction, even at infection levels of less that 20 infected filaments (Leef et al., 2005). The ability of a fish to perform in a swimming performance test (U_{crit}) is associated with the cardiorespiratory performance of the fish, as evidence has shown that individual variation in cardiac output is associated with individual variation in swimming performance. Therefore the effects of AGD on the swimming performance of Atlantic salmon were examined. It was hypothesised that the reduction in cardiac performance observed in previous studies would translate into an appreciable decrease in swimming performance and subsequent recovery. Freshwater bathing has shown be an effective therapeutic agent for alleviating a majority of the gill lesions and reducing viable amoeba numbers in affected salmon. However, what is not known is whether the subsequent reduction in viable amoeba numbers after a freshwater bath would alleviate any potential observable reduction in swimming performance. Therefore a freshwater bath was also incorporated into the swimming performance experiment, with the hypothesis being that the

freshwater bath would mitigate any restriction in swimming performance or recovery.

Chapter 5. The effect of AGD on the protein synthesis rates in the gills of affected Atlantic salmon

One of the major clinical signs of AGD is a pronounced gill epithelial cell proliferation. Histology slides of gills affected by AGD that have been stained with a proliferative cell nuclear antigen (PCNA) stain demonstrated a significant increase in proliferating cells along the basal filamental region of the gill (Adams and Nowak, 2003). Gill cell proliferation involves the production of large number of cells, facilitated by an increase in protein synthesis. Therefore the effect of AGD on protein synthesis rates in the gill was examined. Little work has been done in fish examining the effects of disease on protein synthesis, this chapter provides some of the foundations to which further research can be carried out. The hypothesis was that the observed increase in cell proliferation associated with AGD would result in a tangible increase protein synthesis rates in the gill of affected fish. Furthermore, the extent to which protein synthesis accounts for increase in routine metabolic rate was also determined.

Chapter 6. The effect of replacing dietary fish oil with a stearidonic acid rich oil on metabolic rate and metabolic recovery in seawater Atlantic salmon (*Salmo salar* L.).

Dietary fatty acid (FA) composition has been shown to significantly influence metabolic rate, swimming performance (U_{crit}) and ability to recover after exhaustive exercise in a number of species of fish (Richards et al., 2002; Chatelier et al., 2006). Atlantic salmon *Salmo salar* primarily oxidize carbohydrate and lipids in equal proportions during sustainable exercise (30 – 60% U_{crit}), during non-sustainable exercise (> 90% U_{crit}) carbohydrates are primarily used as a fuel source (Richards et al., 2002). However, during recovery from exhaustive exercise, white muscle tissue of rainbow trout *Oncorhynchus mykiss* primarily oxidizes lipids to fuel creatine phosphate hydrolysis and glycogen synthesis in order to restore cellular levels of ATP in preparation for further exercise (Richards et al., 2002). Whilst carnivorous fish have evolved with fish oil from prey species being the primary source of lipids, current fields of research are examining the effect of replacing fish oils in diets of farmed fish in order to reduce the reliance of the aquaculture industry on marine oils due to high costs and concerns over the sustainability of marine oils. One possible oil replacement is stearidonic acid (SDA), which is a potential precursor oil to the essential fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Some oil replacement diets have shown to have significant effects on the metabolic rate of fish (McKenzie, 2001), thus offering a quantifiable measure of the effect that a replacement may have on the physiology of the fish. Therefore, the effects of SDA on the metabolic rate and metabolic scope where examined to determine whether SDA is a suitable oil replacement for fish oil. The hypothesis for this experiment was that if Atlantic salmon smolt were able to synthesis EPA from SDA, then the increase in

metabolic rate due to insufficient EPA in the diet demonstrated in previous experiments would not occur.

Samples that were used in this chapter were taken from experimental animals that were being used in another research experiment that was examining whether SDA was a suitable candidate as fish oil replacement in Atlantic salmon smolt. Further information can be gathered from Miller thesis, 2008, and Miller et al., 2007a,b.

Chapter 2 – The effect of an acute necrotic bacterial gill infection and feed deprivation on the metabolic rate of Atlantic salmon *Salmo salar*

2.1 INTRODUCTION

Stressors, including disease, impose a metabolic cost on fish that consists of two mechanisms; (1) an energy requirement to manage the disturbances associated with the stressor and (2) an energy cost related to correcting the associated ionoregulatory imbalance (Barton and Iwama, 1991). Measurements of oxygen consumption rates, which are essentially an indirect measurement of metabolic rates (MO_2) (and will be referred to as such), are used to evaluate the amount of energy an organism is using at any given moment (Jobling, 1994). The metabolic rate of most animals fluctuates between two extremes, with the lower limit typically referred to as standard or basal metabolic rate ($MO_{2\ basal}$) and is the metabolic rate of a quiescent animal in a post-absorptive nutritional state, below which physiological function is impaired (Brett and Groves, 1979; Jobling, 1994). However, fish ordinarily expend energy above this level, due to activities such as feeding and locomotion, and this lower level is referred to as routine metabolic rate ($MO_{2\ rout}$). The upper extreme is referred to as active or maximum metabolic rate ($MO_{2\ max}$) and the range through which the aerobic metabolic rate can vary is regarded as the scope for activity or metabolic scope ($MO_{2\ max} - MO_{2\ basal}$) (Fry, 1947).

Alternatively, metabolic scope can be expressed as relative metabolic scope, which is the difference between the routine and maximum metabolic rate (Wieser, 1985).

Metabolic rate measurements are highly sensitive and can vary due to a number of factors including fish size (Hunt von Herbing and White, 2002), nutritional history of the fish (Jobling, 1981; Carter and Brafield, 1991; Shoemaker et al., 2003), prior stresses (Lankford et al., 2005) and environmental changes, such as temperature and salinity (Schurmann and Steffensen, 1997; Morgan and Iwama, 1998). Recent studies have begun to highlight the link between infection status and metabolic rate, suggesting that metabolic rate measurements might be an effective tool for quantifying the impact of disease on fish (Wagner et al., 2003; Tierney et al., 2005; Powell et al., 2005a; Wagner et al., 2005). However the impact of gill diseases on the metabolic rate of Atlantic salmon has had little attention. A recent study that examined the effect of amoebic gill disease on Atlantic salmon found that salmon in response to the disease had significantly elevated routine metabolic rates whilst being able to maintain maximum metabolic rate despite a perceived reduction in gill surface area (Leef et al., 2007). However, little information exists with regard to the effect that bacterial infections, particularly necrotic bacterial gill infections, have on metabolic rate and metabolic scope.

Tenacibaculum maritimum (formerly *Flexibacter maritimus*) causes a skin infection resulting in ulcerative dermatitis, although gill infections with a necrotizing branchitis are not unusual, with experimental infections progressing similarly to natural infections (Handlinger et al., 1997). This disease affects a number of wild and cultured species including Atlantic salmon *Salmo salar*,

greenback flounder *Rhombosolea taprina*, striped trumpeter *Latris lineata*, red sea bream *Pagrus major*, black seabream *Acanthopagrus schegeli*, Japanese flounder *Paralichthys olivaceus* and rock bream *Oplegnathus fasciatus* (Baxa et al., 1986; Wakabayashi et al., 1986; Handlinger et al., 1997). Atlantic salmon experimentally infected with *T. maritimum* were unable to maintain homeostatic regulation of blood plasma osmolality, resulting in significant increases in blood ion concentration (Powell et al., 2004). In marine teleosts, osmoregulation represents a large proportion of the energy expenditure associated with routine metabolic rate (Jobling, 1994), thus any disturbance due to disease may significantly increase the routine metabolic rate of a quiescent fish.

The routine metabolic rate of Atlantic salmon has been shown to decrease significantly during periods of feed deprivation (O'Connor et al., 2000). The reduction in routine metabolic rate may be due to changes in the biochemical composition of the organs, relative organs weights, changes in protein turnover rates, and reduced immune function (Jobling, 1994; Lim and Klesius, 2003). Withholding feed has also been shown to be a successful strategy for disease management for some diseases (Shoemaker et al., 2003). For example, feed deprived Atlantic salmon had a lower cumulative mortality of 2% compared to 36% mortality in fed animals when experimentally challenged with a pathogenic bacteria *Vibrio salmonicida* (Damsgard et al., 1998). Conversely, feed deprivation has been shown to increase mortality in channel catfish *Ictalurus punctatus* challenged with the bacteria *Edwardsiella ictaluri* (Lim and Klesius, 2003) and *Flavobacterium columnare* (Shoemaker et al., 2003).

The objective of the present study was to determine the combined effects of an acute necrotic bacterial gill infection, using *Tenacibaculum maritimum* as a model, and nutritional deprivation on the routine and maximum metabolic rates and subsequent metabolic scope.

2.2 MATERIALS AND METHODS

2.2.1 **Fish husbandry**

Atlantic salmon smolts with a mean mass of 68.4 g (± 1.7 g SEM) and a mean fork length of 22.0 cm (± 1.1 cm SEM) were obtained from a freshwater commercial hatchery in Tasmania (Saltas, Wayatinah, Tasmania, Australia) and acclimated over two weeks to full strength seawater (35 ‰, 1 µm filtered) at 16°C (± 1°C, mean ± range) in a rectangular fibreglass Rathbun tank (4000 L). Previous experiments conducted at this facility have shown that this is a sufficient amount of time to ensure a majority of the fish smolt correctly.

All experiments were conducted in a separate temperature-controlled room (17°C) that housed four individual 400 L recirculation systems. Each system consisted of a 200 L conical bottom tank and a 200 L sump, which contained a biofilter and mesh to remove solids and catch trap feed. One hundred salmon (25 per tank) were removed from a holding tank and anesthetized using clove oil (0.003%, 90% active ingredient eugenol, Langford sales and Marketing, Margate). A 29 gauge needle was used to make a sub-dermal injection of Alcian blue dye into one of six defined positions on the abdomen of the fish to enable individual

identification, with 16 fish tagged for each tank (O'Connor et al., 2000). Fish were acclimated and fed/starved over a 3 week period, all fish were fed for one week in order to establish a feeding response, after which fish in two tanks were fed twice daily to apparent satiation for a further two weeks, whilst the remaining two tanks had their feed withheld. Water quality was measured daily throughout the experiment, dissolved oxygen remained above 95% saturation, total ammonia levels peaked at 2 mg L^{-1} five days after transfer from the holding tank to the experimental system and was below 0.5 mg L^{-1} at the beginning of the first metabolic rate measurement.

2.2.2 Metabolic rate measurements

Pre-inoculation routine and maximum metabolic rates were sampled following the two-week feeding or withholding period, post-inoculation metabolic rates were taken 30 h after inoculation with bacteria (see below). Food was withheld for 24 h prior to metabolic rate measurements to ensure that fish were in a post-absorptive state to avoid confounding results with specific dynamic action (Jobling, 1994). Fish were placed into respirometry boxes 16 h prior to measurement. The respirometry boxes were connected to two separate 150 L recirculation systems, with nine boxes per system, and inlet water into the respirometry boxes was 100% air saturated.

A thermostatically controlled oxygen electrode (1302 electrode, Strathkelvin instruments Ltd Glasgow, UK) connected to a Strathkelvin instruments model 782 O_2 meter was calibrated using a 2% $NaSO_3$ (zero) solution and air saturated

seawater (155 mm Hg), prior to the metabolic rate measurements. To determine routine metabolic rate ($MO_{2\ rout}$), water and airflow to the boxes was halted, and a 3 mL sample of water removed from the box and injected into the oxygen electrode. After 10 min, the water in the box was mixed by pumping the 3 mL syringe 10 times in order to obtain a homogenous water mixture, after which a final water sample was taken and the oxygen content re-measured using the electrode (Powell et al., 2005a).

Maximum metabolic rates ($MO_{2\ max}$) were measured using protocols similar to that found in Cutts et al. (2002). Briefly, individual fish were removed from respirometry boxes and placed into a 50 L cylindrical container that contained hyperoxic seawater (120% air saturation, 17°C), the salmon where chased by hand to exhaustion (10 min). Fish were immediately returned to the 1 L boxes and their oxygen consumption rate measured (as above). Metabolic rate was calculated using the following formula:

$$MO_2 = \frac{((PO_{2i} - PO_{2e}) * \alpha) * V}{T * M} \qquad [1]$$

where PO_{2i} and PO_{2e} are the initial and final oxygen tensions respectively (mm Hg), α is the molar O_2 solubility in water ($\mu M\ O_2\ L^{-1}\ mm\ Hg^{-1}$), V is the respirometer box volume (L), T is the time between the initial and the final oxygen measurements (s) and M is the mass (g) of the fish (Cameron, 1986; Cech, 1990). There was not a complete seal between the air-water interface, the oxygen transfer rates were found to be 0.183 mmHg over a 10 min period, the results were corrected

accordingly. Maximum metabolic rate was measured immediately after net ammonia excretion rates were determined (see below). Routine and maximum metabolic rates and net ammonia excretion rates were re-measured (as described) 30 h post-inoculation.

2.2.3 Net ammonia excretion rates

Net ammonia excretion rate measurements were performed between the $Mo_{2\ rout}$ and $MO_{2\ max}$ determinations. Water flow to the respirometer boxes was stopped and a 3 mL water sample was extracted and immediately frozen at -20°C. After 1 h a second water sample was taken, frozen and water flow was resumed. Water samples were analysed using methods described by Verdouw et al (1978). Excretion rates were calculated using the following formula:

$$NH_4^+{}_{flux} = \frac{([NH_4^+]_{final} - [NH_4^+]_{initial}) * V}{M} \qquad [2]$$

where $[NH_4^+]$ is the concentration of ammonia (μmol L^{-1}), V is the volume (L) of the respirometry box and M is the mass of the fish (g). Air was supplied throughout the experiment with dissolved oxygen levels remaining above 95% saturation for the duration of the experiment.

2.2.4 Inoculation

Cultures of T. maritimum strain 00/3280 were obtained from cultures held at the Tasmanian Aquaculture and Fisheries Institute (Fish Health Unit, Department of

Primary Industries and Water, Tasmania, Australia). Isolates were originally isolated from farmed trout raised in seawater and identified using a 16S ribosomal RNA (rRNA) PCR primer set specific for T. maritimum, cultures were subsequently stored at -80°C on MicroBank beads (Pro-Lab Diagnostics) for later use (Powell et al 2004). Briefly, 200 mL of Shieh's medium formulated with seawater mineral salts buffer (MSB) was inoculated with T. maritimum and gently agitated at 20°C for 48 h. The suspension was centrifuged at 2500 RCF, the pellet washed twice and resuspended in 15 mL of sterile seawater, bacterial concentration was determined by serial dilution. Following the pre-inoculation $MO_{2\ max}$ measurement, a 200 μL suspension of T. maritimum (5 x 10^{12} cells mL^{-1}) was applied evenly over all of the eight gill arches of the anaesthetised Atlantic salmon (Powell et al., 2004; 2005b). The 00/3280 strain of T. maritimum has been shown to be highly pathogenic to Atlantic salmon at high concentrations in a previous study (Powell et al., 2004). Furthermore, in Powell et al (2004; 2005b) the infection remained primarily as a gill disease and did not manifest as a skin lesion, as the aim of this particular trial was to use an acute necrotic gill infection as a model for examining the effects on metabolic rate it was deemed appropriate to use a high bacterial concentration similar to that previously described (Powell et al., 2004). Furthermore, it allowed for a relative comparison with regard to the physiological effect of infection on the fish with previous studies conducted (Powell et al., 2004; 2005b). Control fish received a saline solution and all fish were returned to their respective tanks. After 30 h, $MO_{2\ rout}$, $MO_{2\ max}$ and net ammonia excretion rates were re-measured (post-inoculation samples) as described above.

Following the post-inoculation metabolic rate measurements, fish were given a lethal overdose of clove oil (0.005%), weighed and fork length measured. Three millilitre blood samples were taken via a caudal puncture and were centrifuged at 8000 RCF for 2 min using a Spinwin MC – 01 (Tarsons Products Pty. Ltd) and the plasma was decanted and frozen (-20°C). Blood plasma osmolality was measured using Wescor Vapro 5520 vapour pressure osmometer (Helena Laboratories, Beaumont, Texas). Additionally, a sterile plastic loop was used to sample mucus from the gills and plated on Shieh's marine agar to determine the presence of *T. maritimum*. Cultures were incubated at 20°C for 36 h, colonies were confirmed as *T. maritimum* by colony colour and shape (Wakabayashi et al., 1984; Powell et al., 2005b). This was considered sufficient as the experiment was following protocols used in Powell et al. (2005b) in which colony colour and shape were used primarily as a means of identifying *T. maritimum*. Furthermore, the aim of this particular experiment was to determine the effect of gill necrosis on metabolic rate rather than examining the specific effects of *T. maritimum* per se, therefore identification past colony shape and colour was deemed unnecessary. The entire gill was excised and rinsed gently in seawater and placed into saltwater Davidson's fixative for 24 hours, then into 70% ethanol for histology (Nowak and Adams, 2003). The entire stomach from the end of the oesophagus to the beginning of the pyloric caeca was removed and placed in 10% buffered formalin. Twenty-four hours following fixation, the internal organs were transferred into 70% ethanol. The second left anterior gill arch and a thin slice (approximately 3 mm) of a centre section of stomach was removed, dehydrated, embedded in paraffin wax, sectioned at 5 μm and stained with

haematoxylin and eosin (H&E). Gill sections were then viewed at 100x magnification for signs of focal branchial lamellar necrosis and associated bacterial mats. Stomach cross-sections were viewed for signs of feed deprivation such as a thinning of the longitudinal and circular muscle layer and necrosis of the mucosal epithelium.

2.2.5 Statistical analyses

The study was completed in triplicate to obtain statistically significant numbers of fish, so there were 19, 8, 11 and 16 in the fed infected, unfed infected, fed uninfected and unfed uninfected groups respectively. Statistical analyses were conducted using the statistical package SPSS for Windows (Version 11.5). A two-way ANOVA was used to determine whether there was a significant difference in change in weight between the fed treatments (infected and uninfected) and the unfed groups. Infection (infected/uninfected) and feeding (fed/unfed) were treated as fixed factors, whilst change in weight was treated as the dependant variable.. A paired sample t-test was used to determine the difference between $MO_{2\ rout}$ pre- and post-inoculation for each individual group (referred to as fed infected, fed uninfected, unfed infected, unfed uninfected respectively). A similar analysis was used for $MO_{2\ max}$ and metabolic scope. A one-way analysis of variance (ANOVA) was used to determine differences in blood plasma osmolality among the four groups and significant differences were investigated using a Tukey's post-hoc test. For the ammonia flux, paired sample t-tests were used to determine difference

between the treatments pre- and post-inoculation. Furthermore, two one-way ANOVAs were used to determine whether there was a significant difference between the four treatments pre-inoculation and post-inoculation.

2.3 RESULTS

Tenacibaculum maritimum putatively diagnosed by colony shape and colour was recovered from all animals that were exposed to the bacteria and no cultures developed from samples collected from control fish. Infection with *T. maritimum* presented itself as yellowish mucoid patches at the filament tips (Figure 2.1A). Histologically, there was branchial epithelial necrosis, with overlying bacterial mats (Figure 2.1B). Histological examination of the sections of stomach revealed that both fed and unfed groups had signs of feed deprivation that included necrosis of the mucosal epithelium and a general thinning of the longitudinal and circular muscle layers, this was more prevalent in the starved treatments. Furthermore, fed infected fish in the metabolic rate study lost on average 5.90 ± 1.65 g (± SEM) of their initial mass, whilst unfed infected fish lost 14.6 ± 1.33 g (± SEM). For the uninfected treatments the fed group lost an average of 5.93 ± 1.65 g (± SEM) and the unfed group lost an average of 8.61 ± 2.56 g (± SEM). Infection status had no significant effect on change in weight ($P = 0.136$); however feeding had a significant impact on change in weight ($P = 0.030$), with starved fish loosing significantly more weight than fed fish.

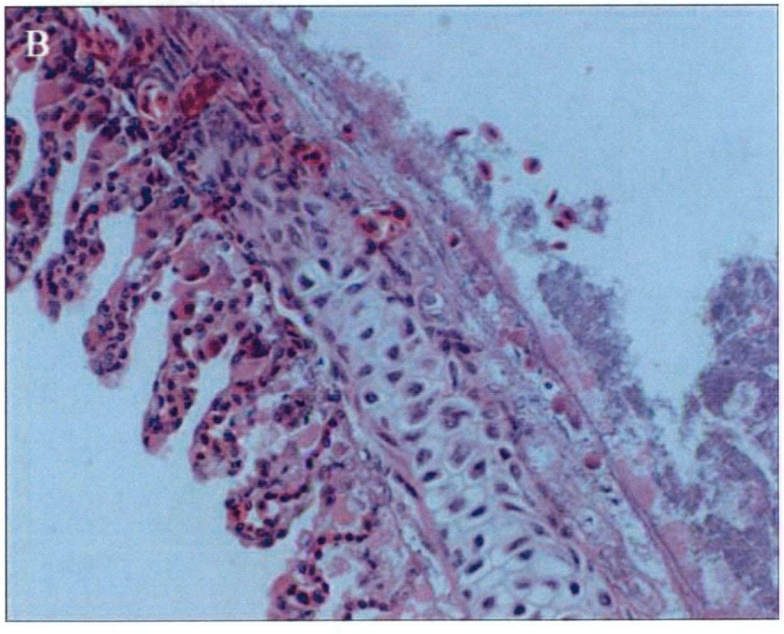

Figure 2.1

(A) Atlantic salmon (*S.salar)* infected with *Tenacibaculum maritimum* showing yellowish mucoid patches on the gill tips due to necrosis. Figure 1 (B). Histological section of gill tissue of Atlantic salmon smolt infected with *T.maritimum.*

Mortalities occurred in all groups with a majority of the mortality occurring immediately after the initial transfer from the seawater acclimation tanks to the experimental tanks, this was attributed to poor conditioned animals that had failed to correctly smolt. Mortalities were as follows; fed infected had a cumulative mortality of 66%, for fed uninfected there was a mortality rate of 24%, starved uninfected had a cumulative mortality rate of 68%, whilst starved uninfected had a cumlative mortality rate of 46%. No mortalities were recorded the week prior to the initial metabolic rate measurement.

2.3.1 Metabolic scope and metabolic rate

The $MO_{2\ rout}$ of Atlantic salmon increased significantly from pre- to post-inoculation levels in infected salmon that were fed and unfed ($P = 0.01$ and 0.02 respectively, Table 2.1). Fed infected fish had an average increase in $MO_{2\ rout}$ of 1.86 ± 0.66 μM O_2 g^{-1} h^{-1} (± SEM) whilst unfed infected fish had an average increase of 2.16 ± 0.72 μM O_2 g^{-1} h^{-1} (± SEM). Maximum metabolic rate increased significantly for unfed uninfected fish ($P = 0.01$, Table 2.1) with an average increase of 2.51 ± 0.61 μM O_2 g^{-1} h^{-1} (± SEM), whilst all other treatment defended $MO_{2\ max}$ from pre- to post-inoculation levels.

Metabolic scope decreased significantly for fed infected fish from pre- to post-inoculation, the reduction represented an overall decrease of 40% from pre-inoculation levels ($P = 0.036$, Figure 2.2). Also, metabolic scope decreased significantly in the unfed infected group with an overall reduction of 49% from pre-

inoculation levels (P = 0.04, Figure 2.2). Uninfected groups had no significant change in metabolic scope.

Table 2.1

Mean (± SEM) routine ($MO_{2\ rout}$) and maximum ($MO_{2\ max}$) metabolic rates (μM $O_2.g^{-1}.h^{-1}$) pre- and post-inoculation for fed infected (n = 19), unfed infected (n = 8), fed uninfected (n = 11) and unfed uninfected (n = 16) Atlantic salmon (*Salmo salar*) exposed to *Tenacibaculum maritimum*. An asterix indicates significant difference between pre- and post-inoculation levels.

	Fed		Unfed	
	Infected	Uninfected	Infected	Uninfected
$MO_{2\ rout}$ Pre	5.00±0.35	6.16±0.55	5.32±0.74	4.32±0.58
Post	6.86±0.54*	5.31±1.10	7.48±0.69*†	5.78±0.38
$MO_{2\ max}$ Pre	10.53±0.53	11.32±0.74	11.08±1.26	8.37±0.43
Post	10.19±0.61	11.89±1.07	10.08±0.84	10.63±0.47*

† one outlier removed due to incomplete water sample.

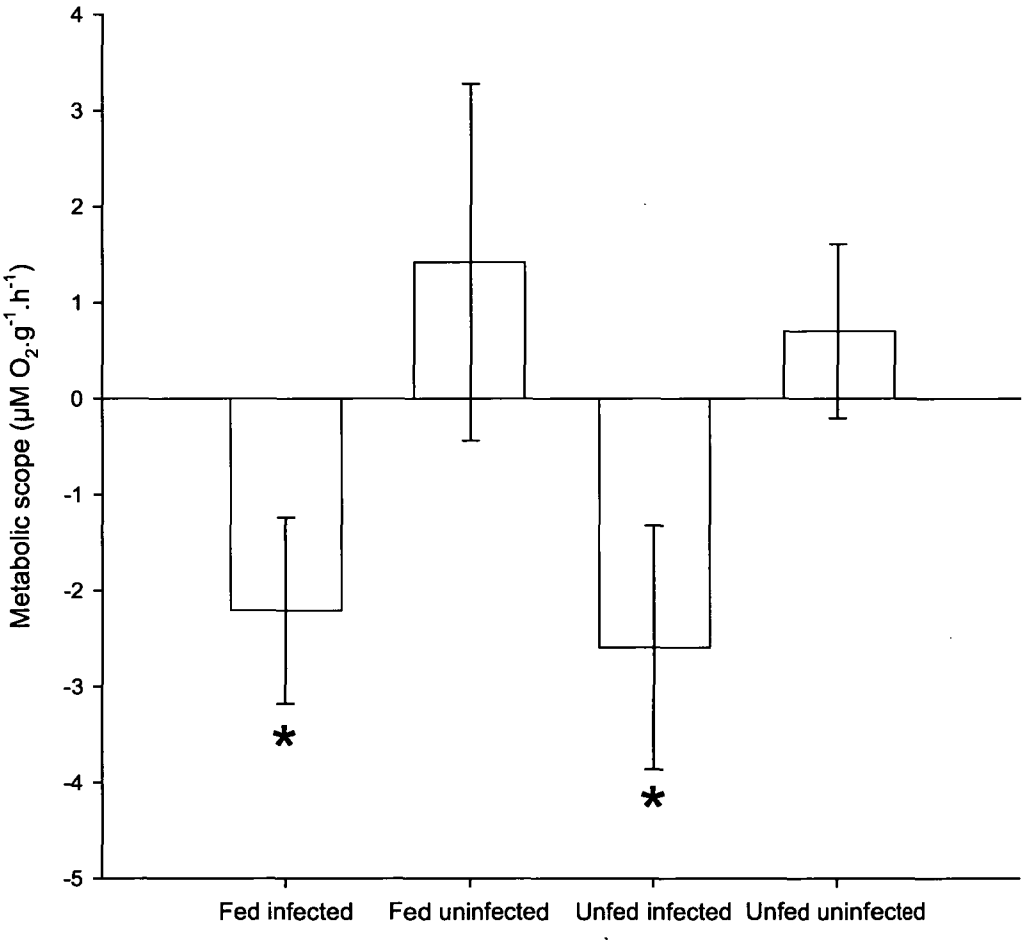

Figure 2.2.

Mean (± SEM) change in metabolic scope from pre to post-inoculation of Atlantic salmon *S. salar* exposed to *T. maritimum*. The asterix indicates a significant change ($P < 0.05$) in metabolic scope from pre- to post-inoculation. The number of fish in each treatment were as follows; fed infected (n = 19), unfed infected (n = 8), fed uninfected (n = 11) and unfed uninfected (n = 16)

2.3.2 Blood plasma osmolality and net ammonia excretion rates

Both infected fed and unfed salmon had significantly higher blood plasma osmolality than uninfected treatments ($P < 0.001$, Figure 2.3A). There was no significant change in net ammonia excretion rates from pre- to post-inoculation for any of the groups. However, a one-way ANOVA showed that there was a significant difference amongst the groups pre– inoculation, with fed infected animals having an average net ammonia excretion rate significantly higher than that of the unfed uninfected treatment ($P = 0.03$, Figure 2.3B).

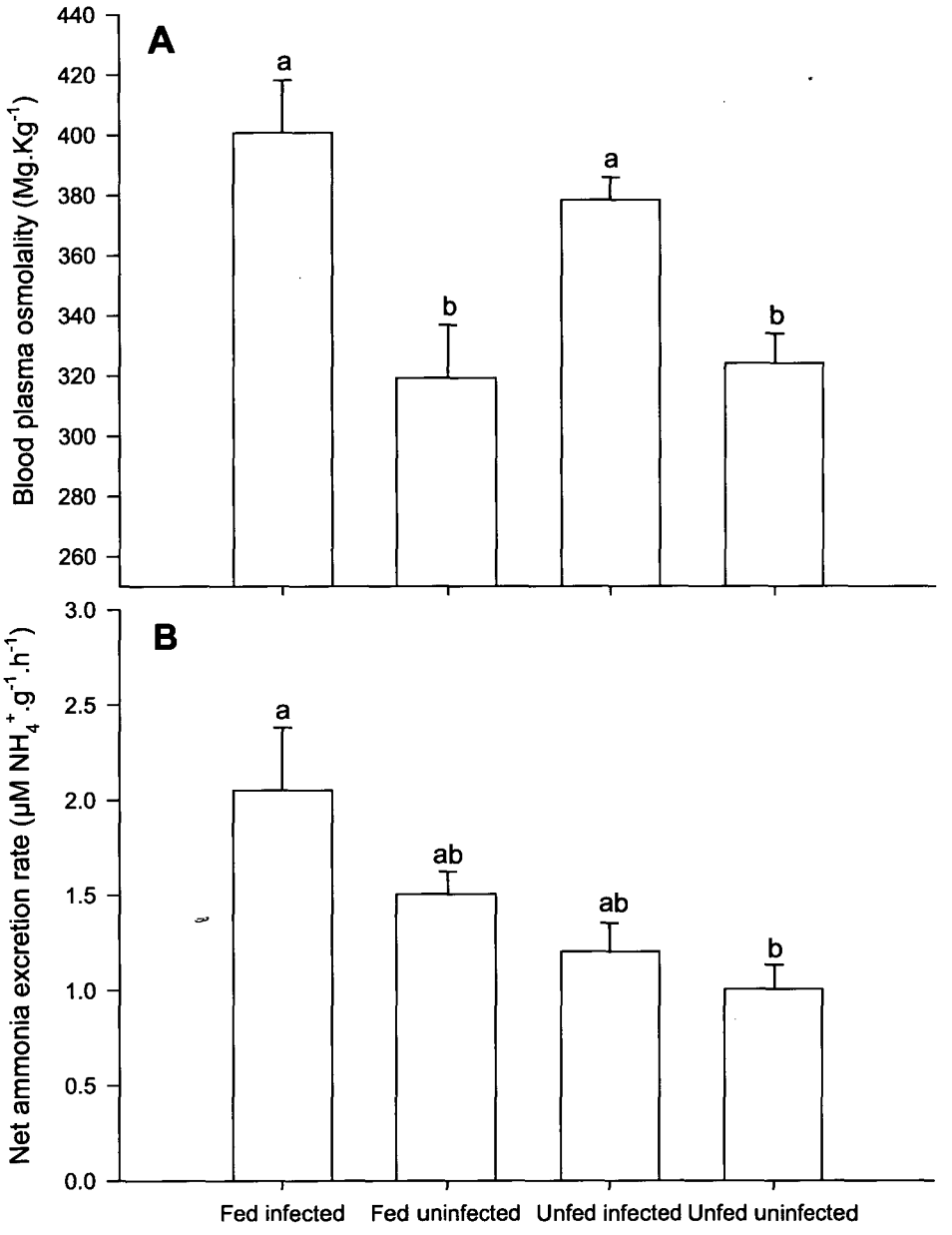

Figure 2.3(A).
Mean (± SEM) blood plasma osmolality of fed and unfed Atlantic salmon *S. salar* exposed to *T. maritimum*. Different superscripts indicate a significant difference among treatments. Figure 2.3 (B). Mean (±SEM) net ammonia excretion rates of Atlantic salmon (*Salmo salar*) pre- inoculation. The number of fish in each treatment were as follows; fed infected (n = 19), unfed infected (n = 8), fed uninfected (n = 11) and unfed uninfected (n = 16).

2.4 DISCUSSION

This study was conducted to test the hypothesis that exposing the gill tissue of Atlantic salmon to *Tenacibaculum maritimum* would result in necrosis of the gill tissue, which would represent a significant additional energetic cost and that this cost could be quantified through changes in metabolic rate. It was expected that infected salmon would spend more energy maintaining osmotic homeostasis as a result of the damage to the respiratory and ionoregulatory epithelium of the gills (Powell et al., 2004). The results showed that the variation in two consecutive MO_2 measurements on the same animal is small and stable enough to allow comparisons between infected and uninfected animals, thus providing a valuable tool for assessing fish health. The major outcome of this study was that necrosis induced by exposure to *T. maritimum* significantly affected metabolic scope and routine metabolic rate, although infected fish were able to defend maximum metabolic rate.

The metabolic scope of Atlantic salmon exposed to *T. maritimum* reduced by 40% to 49% (fed and unfed) from control fish, showing that fish with a necrotic bacterial gill infection have reduced capacity to perform work (Lankford et al. 2005). Furthermore, prior nutritional status did not appear to significantly affect the level of reduction in metabolic scope. To date, limited research has been published examining the effect of bacterial gill infections on the metabolic rate of fish. Of the studies that have focussed primarily on disease and metabolic scope, rainbow trout infected with the parasitic haemoflagellate *Cryptobia salmositica* showed a 44% reduction in metabolic scope (Kumaraguru et al., 1995). Alternatively, rainbow trout infected with *Loma salmonae* successfully defended metabolic scope by

increasing $MO_{2\ max}$, whilst brook trout in the same study decreased $MO_{2\ rout}$ thus increasing metabolic scope in response to infection (Powell et al., 2005a). A decrease in metabolic scope has implications concerning energy allocation by individuals; fish with smaller metabolic scope will inevitably have less energy to allocate to functions such as the replenishment of energy stores growth, reproduction and immune response (Cutts et al., 2002; Lankford et al., 2005).

The decreases in metabolic scope observed in the current study were due to an increase in $MO_{2\ rout}$ for the fed and unfed infected treatments. There are a number of possible explanations for the observed increase in $MO_{2\ rout}$ in infected fish, with the most likely cause being that the additional energy was spent to maintain osmotic homeostasis. This hypothesis was supported by the increase in blood plasma osmolality seen in infected fish. The increase in blood plasma osmolality supports the results found by Powell et al. (2004), in which Atlantic salmon infected by the same strain of *T. maritimum* had an increase in blood plasma osmolality. It is possible that the increase in $MO_{2\ rout}$ was due to an up-regulation of Na^+, K^+ - ATPase as well as other osmoregulatory organs. Na^+,K^+-ATPase is the primary enzyme responsible for maintaining osmotic homeostasis thus any shift in the osmotic equilibrium may result in a significant up regulation of this particular enzyme (Jobling, 1994). However, enzyme activity was not specifically measured in the present study. It is also reasonable to assume that exposure to *T. maritimum* and the subsequent necrosis elucidated a stress response in the fish. Atlantic salmon exposed to *T. maritimum* have significantly elevated plasma lactate levels pre-mortem (Powell et al., 2004), similar results were found for Chinook salmon

Oncorhynchus tshawytscha exposed to *Renibacterium salmoninarum,* which had significantly increased levels of cortisol and lactate, indicating a stress response to infection (Mesa et al., 2000), Increased lactate and cortisol levels in fish have been shown to directly correlate to increases in metabolic rate (Davis and Schreck, 1997). The effect of the transfer of fish from fresh to salt water four weeks prior to the first metabolic rate measurement on routine metabolic rate is suspected to be minimal, as previous studies have shown only a moderate effect on routine metabolic rate (± 5-6%) immediately after transfer (Maxime, 2002).

Maximum metabolic rate for infected Atlantic salmon did not change significantly from pre- to post-inoculation levels, suggesting that the increased costs associated with exposure to *T. maritimum* were not compensated by an increase in $MO_{2\ max}$. The results concur with those found for brook trout infected with *L. salmonae* in that there was no net change in $MO_{2\ max}$ associated with disease, unlike the compensatory increases reported for rainbow trout infected with *L. salmonae* (Powell et al., 2005a). Necrosis due to exposure to *T. maritimum* effectively reduces the available surface area for respiration (Powell et al., 2004). There is evidence to that suggests that in some fish species a reduction in functional gill area is linked to a reduction in $MO_{2\ max}$ (Duthie and Hughes, 1987; Schurmann and Steffensen, 1997). By maintaining $MO_{2\ max}$ despite a reduction in functional gill surface area, salmon in the current study may have employed compensatory mechanisms during infection such as an increased functional surface area of the gills, increasing the permeability of the gill to ions and increased cardiac output (Booth, 1979; Gonzalez and McDonald, 1994; Powell et al., 2000). Indeed salmon infected with *T.*

maritimum do not show signs of hypoxemia or a reduced capacity for the transport of oxygen (Powell et al., 2005b). However, as the cardiorespiratory system in salmonids is perfusion-limited rather than diffusion-limited, any compensatory mechanisms employed must be constrained by the maximum rate that oxygen can be transported through the gill tissue (Powell and Perry, 1999; Gallaugher et al., 2001; Cutts et al., 2002).

Net ammonia flux was shown to be unaffected by disease, which confirmed previous findings (Powell et al., 2004). There was a significant difference between the fed infected and unfed uninfected groups, no significant differences were found for the remaining groups. It was expected that a significant difference would be found between all fed and unfed groups, however, it is possible that the 24 h starvation period prior to measurement that was required for the metabolic rate measurements may have been enough to mitigate the effects of prior feeding. Furthermore, although the fed treatments were supplied with food twice daily to apparent satiation, there was a net decrease in weight over the duration of the experiment suggesting that some of the fish failed to resume feeding upon transfer into the experimental systems. However, the reduction in weight was minimal, and some of this weight loss could be due to the fish being starved for a total of 48 h prior to sampling. Furthermore, the starved treatments lost significantly more weight than the fed treatments thus making the comparisons between fed and starved treatments valid.

The key finding in the present study was an observed decline in the maximum metabolic scope for activity in Atlantic salmon exposed to *T. maritimum*. This was associated with an increase in plasma osmolality, resulting from branchial epithelial necrosis associated with the disease. Interestingly, despite a high degree of necrosis found in the gill tissue, fish maintained maximum metabolic rate. Reduction in metabolic scope has a number of biological implications, the most important being a reduced ability to allocate energy to more desirable outcomes, such as growth and reproduction, which will have profound implications for efficiency in aquaculture.

Chapter 3 - Effect of amoebic gill disease and feed deprivation on the metabolic rate of Atlantic salmon (*Salmo salar*)

3.1 INTRODUCTION

Oxygen consumption rates can be used to indirectly measure metabolic rate (MO_2), and are used to evaluate the amount of energy an organism is using at any given moment (Jobling, 1994). The metabolic rate of most animals fluctuates between 2 extremes, 'standard' or 'basal metabolic rate' (MO_{2basal}) refers to the lower limit and is the metabolic rate of a quiescent animal in a post-absorptive nutritional state, below which physiological function is impaired (Brett and Groves, 1979; Jobling, 1994). However due to activities such as feeding and locomotion fish rarely lower their metabolic rates to such a level, and instead operate at a level referred to as 'routine' metabolic rate (M_{O2rout}),. The upper extreme is referred to as 'active' or 'maximum' metabolic rate (M_{O2max}) and the range through which the aerobic metabolic rate can vary is regarded as the animal's 'scope for activity' or 'metabolic scope' ($MO_{2max} - MO_{2basal}$) (Fry, 1947). Recent studies have begun to highlight the link between infection status and metabolic rate, suggesting that MO_2 measurements might be an effective tool for non-destructively quantifying the impact of disease on fish (Wagner et al., 2003; Tierney and Farrell, 2004; Powell et al., 2005; Wagner et al., 2005). Of the studies that have focused primarily on gill diseases, rainbow trout *Oncorhynchus mykiss* infected with the protozoan parasite *Loma salmonae* showed an increase in MO_{2rout}, while brook trout *Salvelinus fontinalis* infected with the same parasite lowered their MO_{2rout} in response to the

parasitism (Powell et al., 2005). This suggested that the metabolic response to gill-affecting parasites may be host specific (Powell et al., 2005).

Amoebic gill disease (AGD) predominantly affects sea-caged salmonids and is the most significant health issue affecting Atlantic salmon production in Tasmania (Munday et al., 1990). The putative agent is *Neoparamoeba perurans* (Young et al., 2007), the disease manifests as a gill infection and is characterized by multifocal hyperplasic lesions caused by the fusion of lamellae, hyperplasia of the filamental epithelium, accumulation of branchial mucous cells and cellular debris (Munday et al. 1990; Nowak and Munday, 1994). The parasite is present in a number of other countries outside of Australia including Ireland, France, Spain, Chile, USA and New Zealand, as well as being present in a number of host species including rainbow trout *O. mykiss*, coho salmon *Oncorhynchus kisutch*, turbot *Scophthalmus maximus* and seabass *Dicentrarchus labrax* (Munday et al., 1990; Kent et al., 1988; Dyková et al., 1998; reviewed by Munday et al., 2001; Nowak et al., 2002). Mortality from AGD was initially believed to have occurred due to a respiratory disturbance brought on by a reduction in gill surface area, with affected fish showing typical signs of respiratory distress such as lethargy and 'coughing'. However, fish showing AGD lesions do not suffer from an overt hypoxemia (Kent et al., 1988; Powell et al., 2000), and recent evidence suggests that mortality may in fact be related to cardiovascular dysfunction (Powell et al., 2002; Leef et al., 2005a). Atlantic salmon with AGD have significantly elevated systemic vascular resistance and reduced cardiac output, which under stressful conditions could become exacerbated and result in cardiac failure (Leef et al., 2005a). Furthermore,

gas exchange under normoxic conditions has been shown to be only slightly impeded resulting in a respiratory acidosis, suggesting that parasitized Atlantic salmon can compensate for the reduction in effective gill area by redistributing blood flow or changing branchial vascular resistance (Powell et al., 2000; Fisk et al., 2002; Leef et al., 2005b). Clinical AGD was found to have no impact on the MO_{2rout} of Atlantic salmon under normoxic conditions, however under hypoxic conditions AGD affected fish showed a significant reduction in MO_{2rout} and significantly increased mortality (Powell et al., 2000; Fisk et al., 2002). These studies relied on naturally acquired clinical infections; the effects of AGD on MO_{2rout} in a controlled laboratory-based infection have yet to be investigated. A reduction in functional gill area is thought to result in a reduction in MO_{2max} (Duthie and Hughes, 1987), however, no work has been published examining the effects of the reduction of functional gill surface area brought on by AGD on maximum MO_{2max} and metabolic scope.

Anecdotal evidence from farms affected by AGD has suggested that fish with AGD have reduced feed consumption rate with lower growth rates. A reduction in feeding or complete cessation in feeding significantly reduces the MO_{2rout} of Atlantic salmon (O'Connor et al., 2000). A reduction in feed has also been suggested as strategy for disease management for some diseases including *Edwardsiella ictaluri* infection in channel catfish *Ictalurus punctatus* (Wise and Johnson, 1998; Shoemaker et al., 2003). However, the interactions amongst MO_2, nutritional deprivation and disease status have yet to be fully explored. Withholding feed can severely impact some physiological parameters that are associated with

disease resistance, including a reduction in immune response (Lim and Klesius, 2003), reduction in the organosomatic index of organs responsible for immune function (Shoemaker et al., 2003), reduction in MO_2 (O'Connor et al., 2000) and a decrease in protein synthesis rates of some immunoregulatory organs (Smith, 1981).

The main objective of this study was to assess the combined effects of AGD and feed deprivation on the metabolic rates of Atlantic salmon. Furthermore, the hypothesis was tested that infection with *Neoparamoeba* spp., and the subsequent loss of gill surface area, will affect maximum metabolic rate and metabolic scope.

3.2 MATERIALS AND METHODS

3.2.1 Experimental design

Atlantic salmon smolts were obtained from freshwater commercial hatchery in Wayatinah, Tasmania, Australia and acclimated over 2 weeks to 35 ‰, 1μm filtered seawater at 16°C (± 1°C). Dissolved oxygen remained above 95% saturation, and NH_4^+ was at 0.25 mg l^{-1} for the duration of the experiment.

Experiments were conducted in 2 separate recirculation systems consisting of 3 tanks each, with 2 out of 3 tanks in each system used to house fish. The entire experiment was completed twice in order to minimize the impact of individual variation within the tanks on the results. Within each recirculation system one tank of fish was fed on a maintenance ration (MR), the other was unfed (UF). Fish were fed a 3mm commercial pellet obtained from Skretting, Tasmania. For each experimental run, one system had amoebae (see amobae isolation) added, the other

remained amoebae free. Each system consisting of 3 individual 590 l tanks, a 500 l sump and a 500 l header tank giving a total volume of 2770 l. Temperature was maintained at 17°C (± 1°C) via a heat exchange unit passing through an external sump. In order to determine the mass distribution, 150 salmon were weighed and all fish used in the experiment were selected based on mass to be within 1 standard deviation of the mean mass (66.9 ± 17.7g; mean ± SD).

Fish were removed from the holding tank and anesthetized in clove oil (0.0005%), they were then weighed and measured, forty-five fish were then placed into each of the experimental tanks. Sixteen of the 45 fish were tagged using a 29-gauge needle to make a sub-dermal injection of Alcian blue dye into one of 6 defined positions on the abdomen of the fish, thus enabling individual identification. The remaining untagged fish were lethally sampled throughout the experiment for histological analysis (see below). Each tank contained 45 salmon and were fed for 1 week (3 mm pellet, Skretting, Hobart, Tasmania, Australia), after which, 2 tanks of fish continued to be fed twice daily for a further 2 weeks, whilst the remaining 2 tanks had their feed withheld.

3.2.2 MO_2 measurements

Pre-inoculation MO_{2rout} and MO_{2max} samples were taken following the 2-week feeding or withholding period, post-infection metabolic rates were taken 10 and 20 d post-inoculation (see below). MO_2 measurements were conducted in 18 black acrylic boxes with a mean (± SEM) volume of 2153 ± 13 ml. The tagged fish were recorded prior to placement into the black respirometry boxes, the same fish were

then re-measured 10 and 20 days post-inoculation. Eighteen fish from the MR infected group were used in the experiment, 16 fish in the MR uninfected group, 11 in the UF infected group and 14 in the UF uninfected group. Food was withheld for 24 h prior to MO_2 measurements to ensure that fish were in a post-absorptive state (Jobling, 1994). Fish were placed into respirometry boxes 16 h prior to measurement. Water was supplied to the respirometry boxes via a 400 l recirculation system, water temperature was maintained at 17°C ± 0.5°C (mean ± range) with a heat exchange unit, oxygen was maintained at 100% air saturation by an air stone in the header tank. Respirometry boxes were semi submerged in order to maintain a stable temperature when water flow was stopped during metabolic rate measurements.

Prior to all metabolic rate measurements, a thermostatically controlled oxygen electrode (1302 Electrode, Strathkelvin Instruments Ltd Glasgow, UK) was calibrated using a 2% $NaSO_3$ (zero) solution and air saturated seawater (155 mm Hg). The electrode was attached to a Strathkelvin Instruments model 782 O_2 meter. To determine MO_{2rout}, water and airflow to the boxes was stopped and the box sealed. A 3 ml sample of water was removed from the box and injected into the oxygen electrode. After 10 min a final water sample was taken and the oxygen content re-measured using the same electrode (Powell et al., 2005).

$MO_{2\,max}$ was measured by placing individual fish into a 50 l cylindrical tank that contained hyperoxic seawater (120% saturation, 17°C), measured using a Handy Gamma Oxy Guard, (Birkrød, Denmark), the salmon were chased by hand to exhaustion (10 min), and immediately returned to the respirometer boxes and their

oxygen consumption rate measured (as above) (Cutts et al., 2002). MO_2 was calculated using the following formula:

$$MO_2 = \frac{((PO_{2i} - PO_{2e}) * \alpha) * (V - M)}{T * M} \qquad (1)$$

where pO_{2i} and pO_{2e} are the initial and final oxygen partial pressures respectively (mm Hg), α is the molar O_2 solubility in water ($\mu M\ O_2\ l^{-1}$ mm Hg $^{-1}$), V is the respirometer box volume (l), T is the time between the initial and the final oxygen measurements (s) and M is the mass (g) of the fish (Cameron, 1986; Cech, 1990). Metabolic scope was calculated by subtracting MO_{2rout} from MO_{2max}. A blank control box was run in which no fish was placed to correct for bacterial respiration, in each case this was below the detectable limits for the oxygen electrode. There was not a complete seal between the air-water interface. In order to determine oxygen transfer-rates the oxygen level in the boxes (n = 18) were reduced to 0 mg l^{-1} with a solution of sodium sulphite at 7.88mg l^{-1} per mg l^{-1} O_2 to be removed. Oxygen measurements were then taken every hour over a 24-hour period and the oxygen transfer rates calculated (results not shown), the oxygen transfer rates were found to be 0.183 mm Hg over a 10 min period and results were corrected accordingly. MO_{2rout} and MO_{2max} were re-measured 10 and 20 days post-inoculation.

3.3.3 *Neoparamoeba* spp. challenge

Neoparamoeba spp. were isolated using the techniques described by Morrison et al. (2004) modified from Howard and Carson (1994) and Powell and Clark (2003). Briefly, gills of post-mortem Atlantic salmon experimentally infected by cohabitation were excised and placed into a container with 0.2 μm filtered seawater. Individual gill arches were separated and placed into a 50 ml centrifuge container with distilled water and gently agitated for 2 min. The preparation was centrifuged at 4000 g for 5 min and the supernatant was discarded. The pellet was re-suspended in 0.2 μm filtered seawater, agitated and the liquid poured into petri dishes (approx 20 ml^{-1} dish) while being careful not to pour out the gill debris. The process was repeated four times, the petri dishes were then incubated for 1 h at 18°C. After 1 h, the liquid in the petri dishes was decanted and placed onto additional dishes and after 1 h, all petri dishes were rinsed and filled with 0.2 μm filtered seawater and incubated for 24 h at 18°C. Amoebae were harvested by decanting the filtered seawater off the petri dishes, 1 ml of trypsin-EDTA (0.05% trypsin) (Invitrogen, Australia) was added. The dishes were gently tapped against the workbench in order to dislodge the amoebae. The supernatant was removed and placed into centrifuge tubes and spun at 4000 g for 5 min, the supernatant drawn off and the pellet resuspended in 0.2 μm filtered seawater. Amoebae numbers were counted using a haemocytometer. Amoebae were introduced directly into the recirculation systems to give a final concentration of 300 cells l^{-1}.

3.3.4 Data collection

Nine non-tagged fish from each of the replicate tanks were lethally sampled on the day of infection (day 0), 10 and 20 days post-inoculation for histological

analysis (see below), whilst tagged fish (MR infected n = 18, MR uninfected n = 16, UF infected n = 11, UF uninfected n = 14) were lethally sampled after the final MO_2 measurement on day 20. An uneven number of fish were used for the different treatments due to the mortality of some of the tagged fish during the experiments. Fish were given a lethal overdose of clove oil (0.05%), after which they were weighed and measured. Gills were excised from the fish and rinsed gently in seawater to remove excess blood before being placed into saltwater Davidson's fixative for histological assessment of AGD severity (Adams and Nowak, 2001). The number of filaments exhibiting AGD lesions were counted and expressed as a proportion of the total number of filaments counted (Adams and Nowak, 2001; Parsons et al., 2001). Furthermore, the lesions present were counted and expressed as the total number of lesions per filament. A filament was only counted when the lamellae were of equal length bilaterally and present to near the tip of the filament, and the central venous sinus was visible for at least two-thirds of the filament (Speare et al., 1997).

3.3.5 Statistical analyses

Statistical analyses were conducted using the statistical package SPSS for Windows (Version 11.5). As the complete experiment was performed twice, a two-way analysis of variance (ANOVA) was used to determine significant differences between experimental runs, with change in $MO_{2\ rout}$, $MO_{2\ max}$, metabolic scope and the mass of the fish set as the dependant variable and the experimental run (1 or 2) set as the fixed factor. To determine whether there was a significant change in mass

over time a one-way analysis of variance (ANOVA) was used for each of the treatments (MR infected, MR uninfected, UF infected, UF uninfected). If significant differences were found ($P < 0.05$), a Tukey's post-hoc test was used to elucidate the nature of the differences.

A 1-way ANOVA was used to assess the progression of the number of percent lesioned filaments for the MR and UF infected groups over time, with time and number of lesions per filament as fixed factors respectively. A Tukey's post-hoc multiple comparisons test to was used to assess potential differences relating to treatment over time.

To assess the differences relating to disease status and nutritional status over time, change in MO_{2rout} and MO_{2max} and metabolic scope from the day of inoculation for each treatment (MR infected, MR uninfected, UF infected and UF uninfected, respectively) were subjected to a repeated measures ANOVA. Significant differences were investigated with a Dunnett's planned contrasts post-hoc test to compare the initial pre-infection time point to each subsequent time point. To compare across groups a 1-way ANOVA was used for each of the three time points followed by a Tukey's post-hoc multiple comparisons test to assess potential differences relating to treatment.

3.4 RESULTS

All salmon that were exposed to *Neoparamoeba* spp. developed clinical signs of AGD. The percentage of filaments with lesions increased throughout the trial

(Figure 3.1), with no significant difference in the percent of filaments with lesions or the number of lesions per filament between the MR and UF infected groups. There was no significant change in the percent of filaments with lesions or the number of lesions per filament from day 0 to day 10 for both infected groups, however both groups had significant changes in the percent of filaments with lesions and the number of lesions per filament from day 10 to day 20 ($P < 0.001$).

3.3.1 Mass

There was no significant change in mass from day 0 over the course of the infection for the MR infected ($P = 0.453$) and MR uninfected ($P = 0.324$) treatments (Table 3.1). Both the UF infected and uninfected groups had a significant decrease in mass from day 0, with a significant reduction in mass from day 0 to day 10 and from day 10 to day 20 (Table 3.1) ($P < 0.001$ for both groups). Mortality was occurred in all groups, with the majority of fish dying immediately after transfer to saltwater. Cumulative mortality rates for the four treatments were as follows; MR infected = 31%, MR uninfected = 34%, UF infected = 41%, UF uninfected = 35%.

Table 3.1.

Mean (± SEM) change in mass (g) from day 0 of Atlantic salmon (*S.salar*) fed on a maintenance ration (MR) or unfed (UF) infected with amoebic gill disease. Different superscripts ($^{a, b}$) along rows indicate significant differences over time. MR infected n = 18, MR uninfected n = 16, UF infected n = 11, UF uninfected n = 14.

	Day 10	Day 20
MR infected	2.7 ± 2.1^{a}	-1.2 ± 3.7^{a}
MR uninfected	0.6 ± 2.3^{a}	7.4 ± 3.6^{a}
UF infected	-6.3 ± 0.8^{a}	-14.3 ± 1.2^{b}
UF uninfected	-7.4 ± 1.1^{a}	-11.1 ± 1.1^{b}

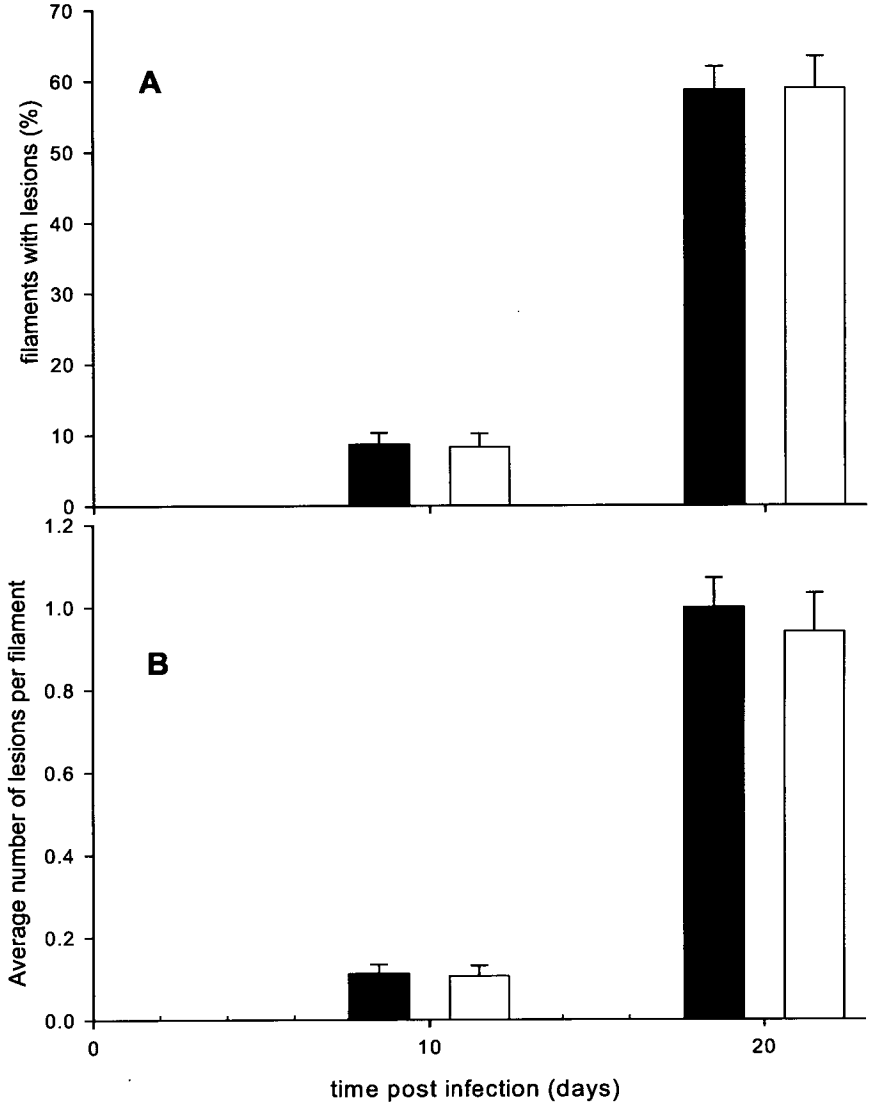

Figure 3.1.

Mean (± SEM) number of filaments with lesions (A) and number of lesions per filament (B) of Atlantic salmon gills exposed to *Neoparamoeba* spp. Black bars represent MR infected fish, white bars represent UF infected fish. Infection levels for both groups (MR and UF) at day 0 was 0% infected filaments and 0 lesions per filament. MR infected n = 18, MR uninfected n = 16, UF infected n = 11, UF uninfected n = 14.

3.3.2 Effect on $MO_{2\,rout}$, $MO_{2\,max}$ and metabolic scope

There was no significant change in MO_{2rout} from day 0 to day 10 for any of the treatments. However, there was a significant increase in MO_{2rout} from day 0 to day 20 for both infected treatments ($P < 0.001$, Figure 3.2A). No significant differences were found between any of the treatments on day 10. On day 20 change in MO_{2rout} of MR and UF infected treatments were significantly larger than both uninfected treatments ($P < 0.001$). There was no significant change in MO_{2rout} for the uninfected treatments.

There were no significant changes in $MO_{2\,max}$ from day 0 to day 10 for any of the treatments. However, there was a significant increase in $MO_{2\,max}$ from day 0 to day 20 for the MR uninfected and both infected and uninfected UF treatments ($P < 0.001$ for all treatments, Figure 3.2B). No significant differences were found between the treatments on day 10 with regard to change in $MO_{2\,max}$ from day 0, however, on day 20 UF infected and UF uninfected treatments had a significantly higher change in $MO_{2\,max}$ from day 0 than the MR infected treatment ($P = 0.004$, Figure 3.2B). There was no significant difference between both MR treatments.

From day 0 to day 10 there was no significant change in scope for any of the treatments, however, from day 10 to day 20 the metabolic scope of the MR uninfected treatment significantly increased, whilst metabolic scope significantly decreased for the MR infected treatment ($P < 0.001$, Figure 3.3). There was no significant change in scope from initial measurements between the groups for day 10. On day 20 the MR infected treatment had significantly decreased more from

day 0 than the uninfected treatments, whilst the MR uninfected treatment had increased significantly more than the UF infected treatment ($P < 0.001$).

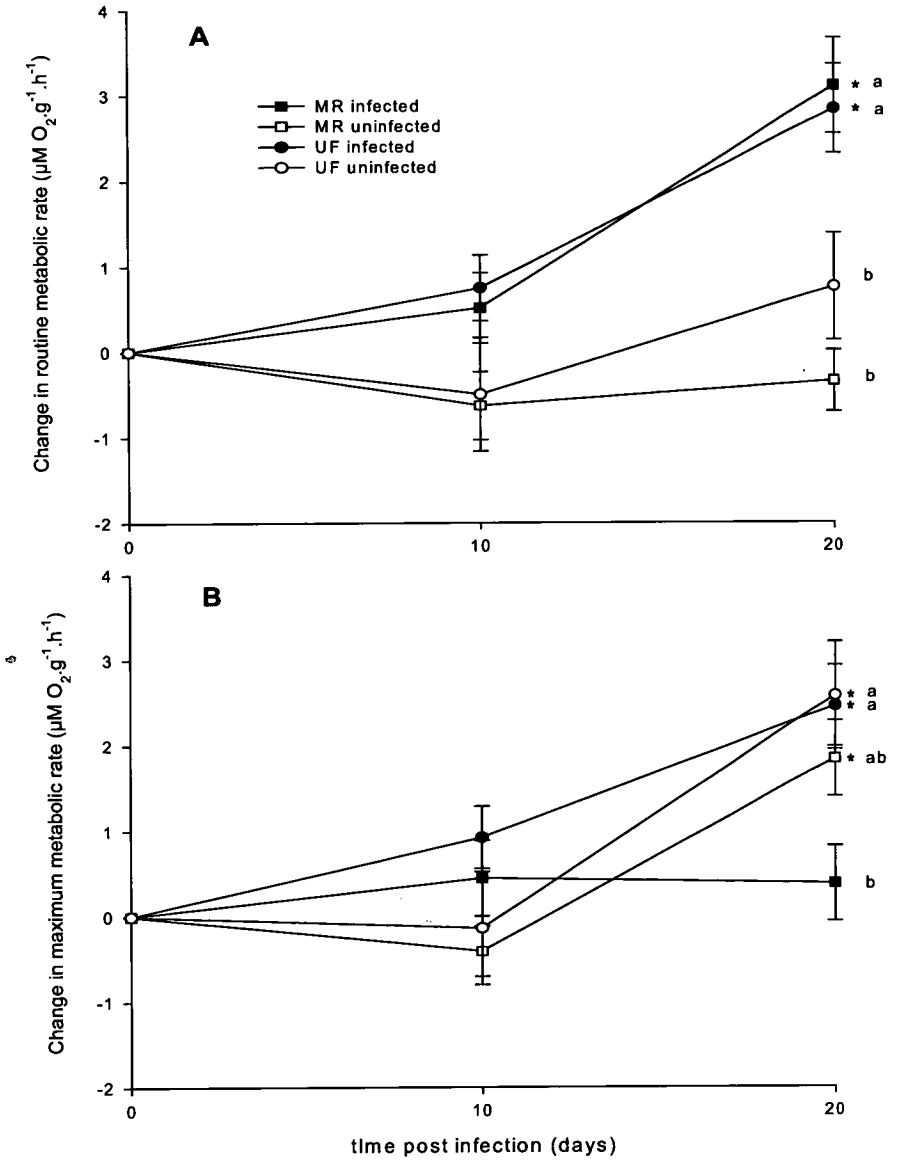

Figure 3.2.

Mean (± SEM) change in routine (A) and maximum (B) metabolic rates from day 0 (time from infection) to day 10 and from day 0 to day 20 of Atlantic salmon infected with *Neoparamoeba* spp. Letters indicate significant differences between treatments on day 20, asterix indicates significant difference between day 0 and day 20. MR infected n = 18, MR uninfected n = 16, UF infected n = 11, UF uninfected n = 14.

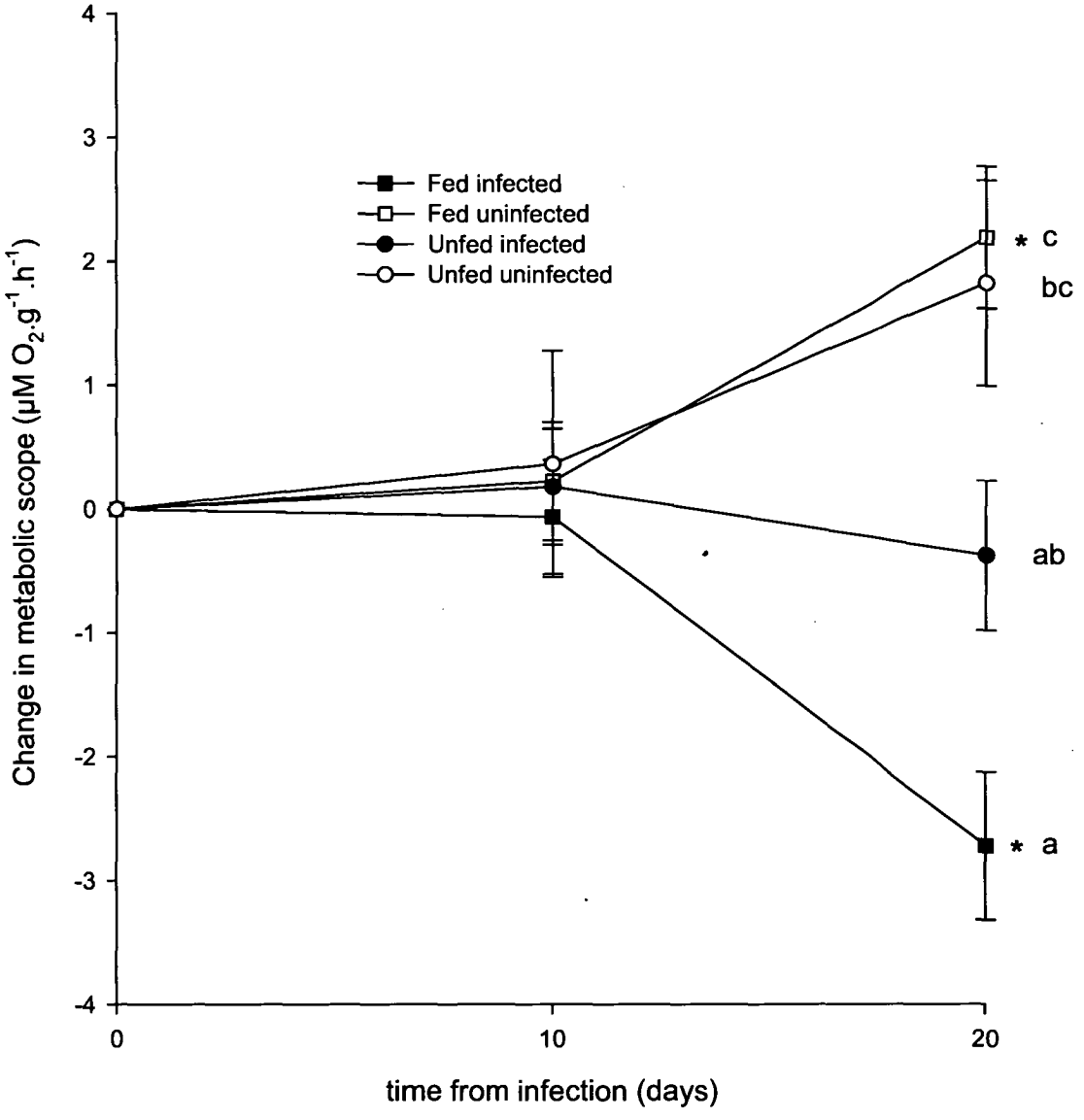

Figure 3.3.

Mean (± SEM) change in metabolic scope from day 0 (time from infection) to day 10 and from day 0 to day 20 of Atlantic salmon exposed to AGD. Letters indicate significant differences between treatments on day 20, asterix indicates significant difference between day 0 and day 20. MR infected n = 18, MR uninfected n = 16, UF infected n = 11, UF uninfected n = 14.

3.4 DISCUSSION

This study tested the hypothesis that the $MO_{2\ rout}$ of Atlantic salmon is significantly affected by *Neoparamoeba* spp., and that prior nutritional history can affect the magnitude of the effect. Furthermore, it was hypothesized that as a result of the reduced gill surface area, parasitized Atlantic salmon would fail to defend $MO_{2\ max}$. The results indicated that AGD had a significant impact on the MO_{2rout} and relative metabolic scope of Atlantic salmon, and the magnitude of the effect was linked to the severity of the infection. No significant difference in disease severity in terms of the number of filaments with lesions and the number of lesions per filament was found between the MR and UF infected groups from the day of infection to 10 days post-infection, however there was a significant increase in severity of infection from day 10 to day 20 for both infected treatments. There was a concomitant increase in the magnitude of the change in MO_{2rout} from initial MO_{2rout} levels for both infected treatments from day 10 to day 20 of the infection, suggesting that the increase in infection load impacted on the MO_{2rout} of Atlantic salmon. There was no significant difference in the change in MO_{2rout} between the MR and UF infected groups, suggesting that prior nutritional status did not affect the severity of change in MO_{2rout}. Previous studies have found that AGD had no significant effect on MO_{2rout} in Atlantic salmon, which contrast to the results found in the current study (Powell et al., 2000; Fisk et al., 2002). However the conflicting results between the current study and the previous studies are likely to reflect the difference in methods employed and the lower infection levels used in the previous

studies. Both of the previous studies focused on chronically infected fish collected on a farm site during a freshwater bathing process, rather than tracking changes in MO_2 over the course of an acute experimental infection, as the present study did. The methods used in the current study are likely to be more sensitive in investigating the effects of disease on MO_2 as the fish were repeatedly measured over the duration of the infection. Alternatively, the differences found between the previous studies and the current study may reflect the differing nature of the infections studied. The fish used in Powell et al. (2000) and Fisk et al. (2002) had what would be regarded as light to medium level infection, as the results of this particular study have shown an effect on MO_{2rout} only becomes apparent at relatively high infection loads.

The blood oxygen tension ($P_a O_2$) of AGD affected salmon has been shown to be significantly less than that of uninfected salmon under normoxic conditions, with the P_aO_2 of infected and uninfected salmon reaching unity under hypoxia (Powell et al., 2000). The gradient of O_2 across the gill and the blood O_2 content determine the O_2 uptake in fish, previous work has found that affected salmon have a decreased P_aO_2 therefore an increase in MO_{2rout} may be associated with a reduction in P_aO_2 (Perry and McDonald, 1993; Powell et al., 2000). However Powell et al (2000) found no significant increase in MO_{2rout} at low infection levels (equivalent to day 10 infection levels in the current study) associated with a decrease in P_aO_2 , and concluded that the reduction in P_aO_2 was not sufficient to reduce blood oxygen content to a level in which an increase in MO_{2rout} would be expected. The higher infection levels found in the current study may however be sufficient to elicit a

reduction in blood O_2 content, thus potentially increasing MO_{2rout}. The increase in MO_{2rout} may also be associated with an increase in protein turnover rate in gill tissue. Infection with *Neoparamoeba* spp. induces a multifocal hyperplastic response in the gill tissue associated with undifferentiated epithelial cell proliferation, as well as a significant increase in mucous production brought on by an increase in the number of mucus cells present on the lamellae (Munday et al., 1990; Nowak and Munday, 1994; Adams and Nowak, 2003; Roberts and Powell, 2003). Cell proliferation, which is essentially tissue growth, requires an increase in protein synthesis, an energetically expensive process. Protein synthesis accounts for 15 –25% of the basal metabolic cost in fish, with gill tissue accounting for the second highest rate of protein synthesis behind the liver (Houlihan et al., 1988, see review Carter and Houlihan, 2001). Gill tissue exposed to an irritant will respond by increasing protein turnover in order to repair damaged tissue (Wilson et al., 1996). However gill tissue only accounts for approximately 2-3% of total body weight, so even a large increase in protein synthesis in the gill tissue is unlikely to result in a significant increase in whole body oxygen demand (Lyndon and Houlihan, 1998). An increase in $MO_{2\ rout}$ may also be associated with an immune response to AGD. The immune-regulatory gene interleukin-1β (IL-1β) has been shown to be up-regulated in the gills of AGD exposed Atlantic salmon, suggesting that there is some involvement of a cellular (non-specific) immune response at the molecular level (Bridle et al., 2006). However, the extent to which Atlantic salmon mount a non-specific immune response to AGD is currently unknown. It is unlikely that any

one cause is behind the increase in $MO_{2\ rout}$, rather it is likely that it is a combination of the factors discussed.

MR infected fish maintained $MO_{2\ max}$ over the duration of the infection, whilst all other treatments significantly increased $MO_{2\ max}$ from day 0 to day 20 of the infection. Contrary to the initial hypothesis that fish with AGD would show a decrease in $MO_{2\ max}$ due to a reduction in gill surface area, fish with AGD seem to be capable of coping with the reduction in gill surface area brought on by infection. It was initially thought that the loss of functional lamellae, which potentially reduces functional gill surface area and increases the diffusive distance between the water and the blood in the gill, would result in a decrease in $MO_{2\ max}$. In rainbow trout, a reduction in functional gill area was linked to a reduction in $MO_{2\ max}$ (Duthie and Hughes, 1987; Schurmann and Steffensen, 1997). However, the current results suggest that AGD affected Atlantic salmon suffer no such effect. Whilst MR infected fish defended $MO_{2\ max}$, there was a significant reduction in metabolic scope due to a significant increase in $MO_{2\ rout}$. These results are in contrast to those found by Powell et al. (2005) for rainbow trout and brook trout infected with the protozoan gill parasite, *Loma salmonae*, where both species defended scope either by increasing $MO_{2\ max}$ (rainbow trout) or decreasing their $MO_{2\ rout}$ (brook trout). It also appears that $MO_{2\ rout}$ in rainbow trout and brook trout responds to infection with *Loma salmonae* at much lower infection levels (1.8 – 2 xenomas per gill arch) than $MO_{2\ rout}$ in Atlantic salmon affected by AGD, which had no $MO_{2\ rout}$ response at infection levels of approximately 10% infected filaments. The results suggest that

the response to gill diseases by salmonids is therefore quite host and disease specific (Powell et al., 2005).

The most important finding for this study was that AGD leads to a significant increase in MO_{2rout}, and that the increase in MO_{2rout} increases with infection severity. Furthermore, we showed that MO_{2max} can be successfully defended, suggesting that affected Atlantic salmon may be employing compensatory mechanisms in response to AGD. Metabolic scope was also shown to be significantly reduced in MR infected fish, but defended in UF infected fish.

Chapter 4 - Effect of amoebic gill disease on the swimming performance and recovery of Atlantic salmon *Salmo salar*

4.1 INTRODUCTION

Amoebic gill disease (AGD) predominantly affects sea-caged salmonids and is the most significant health issue affecting Atlantic salmon production in Tasmania (Munday et al., 1990; Munday et al., 2001). The putative agent is *Neoparamoeba perurans* (Young et al., 2007), the disease is characterized by multifocal hyperplasic lesions caused by the fusion of lamellae, hyperplasia of the filamental epithelium, accumulation of branchial mucus cells and cellular debris (Munday et al., 1990; Nowak and Munday, 1994). A reduction in gill surface area due to the lesions on the gill was initially thought to have induced a respiratory disturbance, with affected fish showing typical signs of respiratory distress such as lethargy and 'coughing'. However, fish showing AGD lesions do not suffer from an overt hypoxemia (Kent et al., 1988; Powell et al., 2000), and recent evidence suggests that mortality may, in fact, be related to cardiovascular dysfunction (Powell et al., 2002; Leef et al., 2005a). Atlantic salmon with AGD have significantly elevated systemic vascular resistance and reduced cardiac output, which under stressful conditions could become exacerbated and cause cardiac failure (Leef et al., 2005a; Leef et al., 2007). Furthermore, gas exchange under normoxic conditions is only

slightly impeded, suggesting that parasitized Atlantic salmon compensate for the reduction in effective gill area by redistributing blood flow or changing branchial vascular resistance (Powell et al., 2000; Fisk et al., 2002; Leef et al., 2005b). However, it is not known to what extent the hyperplastic gill lesions restrict the ability of salmon to obtain oxygen during times of high demand, for example during fast swimming, and whether this may affect the overall swimming performance of the fish.

The ability of a fish to carry out swimming performance tests has proven to be a reliable and effective means of assessing the pathophysiological affects of disease (Wagner et al., 2003; Tierney and Farrell, 2004). A number of recent studies have highlighted the effect that pathogen loading can have on critical swimming speed (U_{crit}). Atlantic salmon infested with sea-lice (*Lepeophtheirus salmonis*) had significantly reduced U_{crit}, and similar results were found during a *Mycobacterium* spp infection in Delta smelt (*Hypomesus transpacificus*) (Swanson et al., 2002; Wagner et al., 2003). More recently, studies have begun to incorporate a second U_{crit} test as a means of assessing the ability of fish to recover from exhaustive exercise, the quotient of the second and the first U_{crit} tests being referred to as the recovery ratio (Tierney and Farrell, 2004). Ideally, a healthy fish should have a recovery ratio of 1 (unity), fish that fall below a recovery ratio of 1 are generally regarded as having failed to have recovered fully. Sockeye salmon (*Oncorhynchus nerka*) infected with the myxosporean kidney parasite *Parvicapsula minibicornis* have significantly reduced recovery ratios (Wagner et al., 2005), similarly, sockeye

salmon heavily affected by a range of pathological conditions were not able to achieve a similar performance during a second U_{crit} (Tierney and Farrell, 2004). However, there is little information available on the effect of gill diseases, specifically AGD, on the swimming performance and subsequent recovery in Atlantic salmon. This is of vital importance as any reduction in swimming performance may affect the ability of a fish to maintain its position in the water column, particular when habituating areas of high flow.

The commercial treatment for AGD in Tasmania is to transfer fish from holding pens into a pen that contains a liner that has been filled with freshwater. After 2 to 3 hr the liner is removed and the fish returned to the underlying cage. Freshwater has been shown to kill amoeba outright and is very effective at resolving the lesions, it has also been associated with a reduction in hypertension in AGD affected fish, and as such bathing is a very effective treatment for AGD (Howard and Carson, 1993; Clark et al 2003, Powell et al., 2002, Roberts and Powell, 2003).

Following exhaustive exercise there is an elevation in metabolic rate (Mo_2) referred to as excess post-exercise oxygen consumption (EPOC) (Gaesser and Brooks, 1984; Lee et al., 2003). The elevation in MO_2 occurs as the fish restores the cellular balance of oxygen and high-energy phosphates, correcting any osmotic shifts and biochemical imbalances in metabolites and is a measure of the anaerobic cost of exercise (Lee et al., 2003). However, little attention has been paid to the possible effects that disease, particularly gill diseases, could have on the ability of a fish to

restore cellular oxygen levels post-exercise (Lee et al., 2003). It is thought that the restrictions imposed by the lesions associated with AGD may alter the ability of Atlantic salmon to respire aerobically during periods of intense exercise, therefore increasing their reliance on anaerobic metabolism during exercise, this in turn would manifest as an increase in EPOC.

The aim of this study was to assess the effect that AGD has on the swimming performance of Atlantic salmon and the ability to perform a second swimming test. Furthermore, the effect of AGD on EPOC was also assessed. A further experiment was conducted to assess the impact of a freshwater bath on the repeat swimming performance of Atlantic salmon affected by AGD.

4.2 MATERIALS AND METHODS

4.2.1 Experiment 1 Effect of amoebic gill disease on the swimming performance of Atlantic salmon

4.2.1.1 Fish husbandry

Atlantic salmon smolts with a mean (± SE) mass of 78.0 g (± 3.6 g) and a mean fork length of 20.8 cm (± 0.4 cm) were obtained from a freshwater commercial hatchery in Tasmania (Saltas, Wayatinah, Tasmania, Australia) and acclimated over two weeks to seawater (35 ‰, 1 μm filtered) at 16°C (± 1°C; mean ± range) in a rectangular fibreglass Rathbun tank (4000 L).

All experiments were conducted in a temperature-controlled room (16°C ± 0.2; mean ± range) that housed four individual 400 L recirculation systems. Each system consisted of a 200 L conical bottom tank and a 200 L sump, which contained a biofilter, and mesh to remove solids and catch uneaten feed. Fifty-six (14 per tank) fish were removed from the seawater acclimation holding tank and placed into the recirculation systems, fish were then fed by hand to apparent satiation twice daily. Water quality was measured daily throughout the experiment, dissolved oxygen remained above 95% saturation, total ammonia levels peaked at 2 mg L^{-1} five days after transfer from the holding tank and was below 0.5 mg L^{-1} thereafter.

4.2.1.2 Neoparamoeba spp isolation and experimental challenge

Neoparamoeba spp were isolated using the techniques described by Morrison et al. (2004) modified from Howard and Carson (1994) and Powell and Clark (2003). Briefly, gills of post-mortem Atlantic salmon that had been infected via cohabitation in an experimental challenge AGD tank were excised and placed into a container with 0.2 μm filtered seawater. Individual gill arches were separated and placed into a 50 ml centrifuge container with distilled water and gently agitated for 2 min. The preparation was centrifuged at 4000 g for 5 min and the supernatant discarded. The pellet was re-suspended in 0.2 μm filtered seawater, agitated and the liquid poured into petri dishes (approx 20 ml^{-1} dish) while being careful not to pour out the gill debris. The process was repeated 4 times, the petri dishes were then incubated for 1 h at 18°C. After 1 h, the liquid in the petri dishes was decanted and placed onto additional dishes and after 1 h, all petri dishes were rinsed and filled

with 0.2 μm filtered seawater and incubated for 24 h at 18°C. Amoeba were harvested by decanting the filtered seawater off the petri dishes, 1 mL of trypsin-EDTA (0.05% trypsin) (Invitrogen) was added. The dishes were gently tapped against the workbench in order to dislodge the amoeba. The supernatant was removed and placed into centrifuge tubes and spun at 4000 g for 5 min, the supernatant drawn off and the pellet resuspended in 0.2 μm filtered seawater. Amoeba numbers were counted using a haemocytometer. Amoeba were introduced directly into the recirculation systems to give a final concentration of 400 cells L^{-1}. Water flow was stopped for a period of 6 h to ensure that the amoebae had sufficient time to attach to the gills of the Atlantic salmon, after which the flow was re-established. Two tanks were randomly selected out of the four tanks of fish and were inoculated using 400 cells L^{-1} with the infection staggered by one week between the two tanks. Two tanks remained uninfected controls.

4.2.1.3 Routine MO_2 and EPOC Measurements

Six control fish were tested first, 3 from each of the uninfected tanks. After which salmon from the first infected tank were used until no fish remained, then fish from the second tank were used until no fish remained. Twenty fish were used throughout the experiment, 6 uninfected controls and 14 fish that had been exposed to AGD with a range of infection levels from each of the two tanks. Metabolic rate measurements were conducted in a 2130 mL black acrylic box that was housed within the temperature controlled room. Food was withheld for 24 h prior to metabolic rate measurements to ensure that fish were in a post-absorptive state

(Jobling, 1994). Twelve hours prior to MO_2 and EPOC measurements fish were anaethetised using a 0.005% solution of clove oil and their fork lengths measured, after which they were immediately transferred into the respirometry boxes. Water was supplied to the respirometry boxes via a 90 L recirculation system, water temperature was maintained at 16°C ± 0.2°C (mean ± range), oxygen was maintained at > 97% air saturation by an air stone in the sump.

Prior to all metabolic rate measurements, a thermostatically controlled oxygen electrode (1302 electrode, Strathkelvin Instruments Ltd Glasgow, UK) was calibrated using a 2% $NaSO_3$ (zero) solution and air saturated seawater (155 mm Hg). The electrode was connected to a Strathkelvin instruments model 782 O_2 meter. To determine routine metabolic rate ($MO_{2\ rout}$), water and airflow to the boxes was stopped and the box sealed. A 3 mL sample of water was removed from the box and injected into the oxygen electrode. After 10 min a final water sample was taken and the oxygen content re-measured using the electrode (Powell et al., 2005).

Maximum metabolic rates ($MO_{2\ max}$) were measured using established protocols (Cutts et al. 2002; Jones et al., 2007). Briefly, individual fish were placed individually into 50 L cylindrical containers that contained hyperoxic seawater (120% saturation, 15°C), measured using a Handy Gamma Oxy Guard, (Birkrød, Denmark), the salmon were chased by hand to exhaustion and immediately returned to the respirometer boxes and their oxygen consumption rate measured (as above) (Cutts et al., 2002). Subsequent MO_2 measurements were taken at 15-minute intervals for the first hour then every hour for 6 hours after chasing to determine

EPOC. After the 6th hour EPOC measurement was taken the fish were transferred via a bucket into the swim tunnel for U_{crit} measurements. Total EPOC (mg O_2 kg^{-1}) was calculated by determining the oxygen cost of recovering from exhaustion and by integrating the area bounded between the recovery curve and MO_{2rout}, and between the time the recovery curve reached MO_{2rout}. Metabolic rate was calculated using the following formula:

$$MO_2\ (\mu M\ O_2.\ g^{-1}.\ h^{-1}) = \frac{((PO_{2i} - PO_{2e}) * \alpha) * (V - M)}{T * M}$$

where PO_{2i} and PO_{2e} are the initial and final oxygen tensions respectively (mm Hg), α is the molar O_2 solubility in water ($\mu M\ O_2\ L^{-1}$ mm Hg^{-1}), V is the respirometer box volume (L), T is the time between the initial and the final oxygen measurements (s) and M is the mass (g) of the fish (Cameron, 1986; Cech, 1990). Metabolic scope was calculated by subtracting MO_{2rout} from MO_{2max}. A blank control box was run in which no fish was placed to correct for bacterial respiration, in each case this was below the detectable limits for the oxygen electrode. There was not a complete seal between the air-water interface, in order to determine oxygen transfer rates the oxygen level in the boxes (n = 18) were reduced to 0 mg l^{-1} with a solution of sodium sulphite at 7.88mg l^{-1} per mg l^{-1} O_2 to be removed. Oxygen measurements were then taken every hour over a 24 period and the oxygen transfer rates calculated (results not shown), the oxygen transfer rates were found to be 0.183 mm Hg over a 10 min period and results were corrected accordingly.

4.2.1.4 U_{crit} measurements

Swimming measurements were performed in a Brett-type 222 L swimming tunnel constructed of PVC and fibreglass connected to a 400 L sump, giving a total volume of 622 L. The swim chamber was 120 cm long, 30 cm internal diameter with a transparent lid to allow observations of the fish. An upstream grid ensured a uniform flow over the cross section of the chamber, a downstream grid ensured that the fish was retained within the swim chamber. A circulating flow of water was generated by a Collins 15 cm variable speed centrifugal pump (3ø, 5.5 kW). The voltage delivered to the motor was controlled by an inverter speed controller (SPEECON 7300PA, Teco Pty. Ltd., Aus) calibrated prior to the experiment using a flow meter (Meister DHTF, Meacon Pty. Ltd., Australia) to deliver controlled water velocities. Swimming velocities were not corrected for the solid blocking effect as the cross-sectional areas of the fish were never more than 10% of the total cross sectional area of the swim tunnel (Jones et al., 1971).

Fish were allowed to habituate to the swim chamber for 1 h in aerated stationary water. After the habituation period, salmon were given a conditioning swim that consisted of an abbreviated step velocity test in order to alleviate the potential training effect observed with naïve fish and to provide an index of performance for the subsequent ramp-critical speed (U_{crit}) tests (Farlinger and Beamish, 1977; Jain et

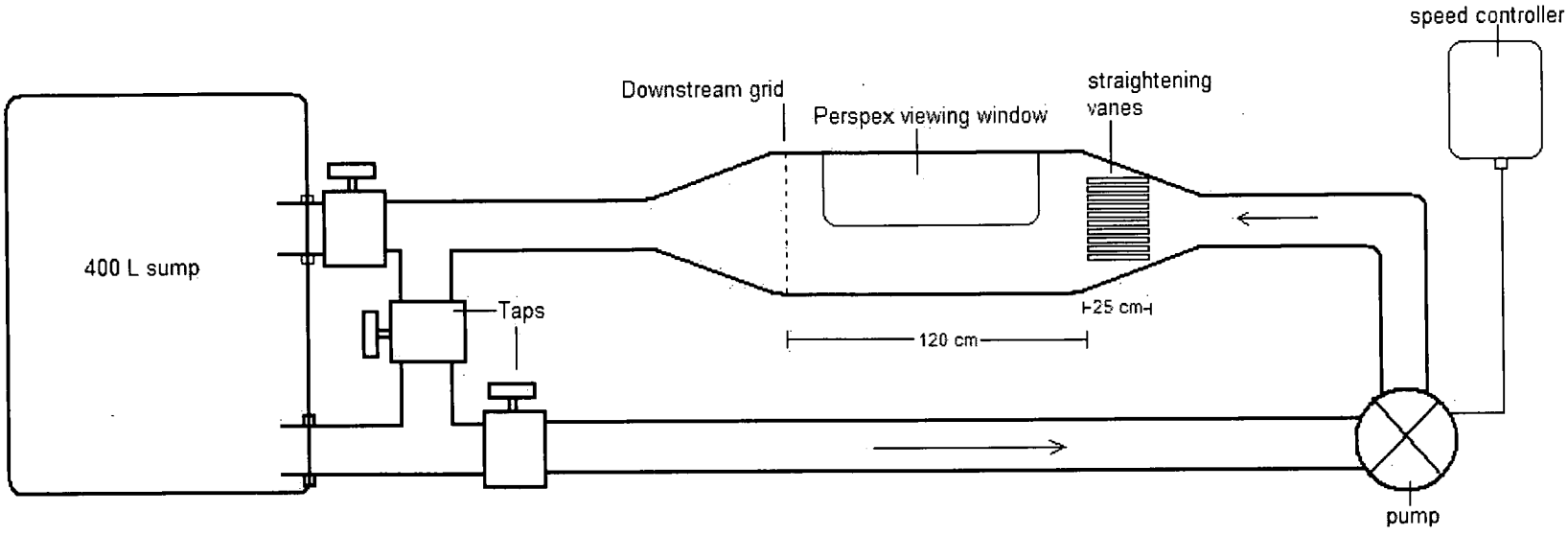

Figure 4.1.

Diagram of the Brett type swim tunnel used to determine swimming performance (Ucrit) for Atlantic salmon affected by amoebic gill disease. Diagram is not to scale.

al., 1997). For the conditioning swim, water velocity was increased in regular increments of 0.15 body lengths per second (BL s^{-1}) every 2 min until the fish rested on the rear grid of the swimming chamber for 15 seconds. Fish were then allowed to recover overnight (16 h) in aerated stationary water.

The following day fish were subjected to a ramp U_{crit} test as described by Jain *et al* (1997). Fish were brought up to 75% of the maximum speed achieved on the conditioning swim in 0.15 BL s^{-1} increments with water velocity increasing every 5 min. Once the fish was at approximately 75% of the conditioning swim water velocity, increments were increased 0.15 BL s^{-1} every 20 min until the fish was exhausted and rested on the rear grid for 10 s. Fish were then allowed a 45 minute recovery period in stationary aerated water, after which a second U_{crit} test was performed using the aforementioned protocol (Tierney and Farrell, 2004). Recovery ratio (RR) was calculated using the formula :

Recovery ratio = $U_{crit\,2} / U_{crit\,1}$

where a value of 1 is regarded as unity, however, a fish was regarded as having fully recovered from the previous swimming performance test if it achieved a RR of more than 0.95 (Tierney and Farrell, 2004). U_{crit} was calculated according to the following equation (Brett, 1964):

$$U_{crit} = U_f + [U_i (t_f / t_i)]$$

where U_{crit} is in BL s^{-1}, U_f is the water velocity of the last fully completed increment (BL s^{-1}), U_i is the water velocity increment (BL s^{-1}), t_f is the time spent at fatigue velocity (min) and t_i is the time period for each completed water velocity increment (min).

4.2.2 Experiment 2. Effect of freshwater bathing on the swimming performance of Atlantic salmon with AGD

All fish husbandry, infections protocols, metabolic rate measurements and U_{crit} measurements were performed as described above except for the following. Fish were stocked at a density of 12 fish per 400 L system, *Neoparamoeba* spp were added to two randomly selected tanks at a density of 400 cells L^{-1} with the infection staggered by 1 week. Eight hours prior to being placed into the swim tunnel, the fish were anaesthetized in 0.03% clove oil and measured. Routine metabolic rates were measured prior to the first U_{crit} measurements as per the previous experiment, $MO_{2\ max}$ and EPOC were not measured. After the first and second U_{crit} tests, fish were given a 2 h freshwater bath (total hardness 34.8 ± 4.6 mg l^{-1} $CaCO_3$; mean ± SEM, 16 ± 0.2°C; mean ± range, 100% oxygen saturation), after which they were placed into a 100 L holding tank that contained seawater (35‰, 100% oxygen saturation). A third and fourth U_{crit} test was performed 48 h after the freshwater bath, after which the fish were lethally sampled (see below). In addition, a saltwater control group was added in which fish were 'bathed' in saltwater (35‰, 100% oxygen saturation) for a period of 2 h in order to determine whether there was any handling effects associated with the bathing process.

4.2.2.1 Data collection

Fish were lethally sampled one hour after the final U_{crit} measurement. Fish were given a lethal overdose of clove oil (0.05%), after which they were weighed and measured. Gills were excised from the fish and rinsed gently in seawater to remove

excess blood before being placed into saltwater Davidson's fixative for histological assessment of AGD severity.

4.2.3 Histology

Twenty-four hours after being placed into the Davidson's fixative, the gill basket was transferred into 70% ethanol. The second left anterior arch was removed, dehydrated, embedded in paraffin wax, sectioned at 5 μm and stained with haematoxylin and eosin (H & E). Sections were then viewed at x100 magnification under a light microscope (Olympus, Hamburg, Germany). The number of filaments exhibiting AGD lesions were counted and expressed as a proportion of the total number of filaments counted (Adams and Nowak, 2001; Parsons et al., 2001). Furthermore, the total number of lesions present were counted and expressed as the total number of lesions per filament (Powell et al., 2002). A filament was only counted when the lamellae were of equal length bilaterally and present to near the tip of the filament, and the central venous sinus was visible for at least two-thirds of the filament (Speare et al., 1997).

4.2.4 Statistics

Mean values ± S.E.M are presented throughout the text and figures unless otherwise stated. The fiducial limit for accepting significance was $P < 0.05$. Statistics were

calculated with SPSS 11.5 statistical software (SPSS, Inc.) and Sigma Plot 8.0 (SPSS Scientific Chicago, IL, USA) was used to generate figures.

For experiment 1, infected treatments were compared to uninfected treatments for routine and maximum metabolic rate, metabolic scope, $U_{crit\ 1}$ and $U_{crit\ 2}$, recovery ratio's, EPOC and time to recovery using an unpaired t-test's. Paired sample t-tests were used to compare $U_{crit\ 1}$ to $U_{crit\ 2}$ for each treatment. Fish with a RR of < 0.95 were defined as having an impaired repeat swimming performance (Tierney and Farrell, 2004, Tierney et al., 2005, Wagner et al., 2005). Infection levels of less than 20% infected filaments are regarded as relatively minor infections, levels above 20% have been associated with significant increases in vascular resistance and decreases in cardiac output, therefore qualitative analysis of RR were divided into fish with less than 20% infected filaments and more than 20% infected filaments (Leef et al., 2005a). Time to recovery was taken as the time it took for metabolic rate to return to that of the routine metabolic rate measurement during the EPOC measurements. For the second experiment, routine and maximum metabolic rate, metabolic scope, recovery ratios (both pre- and post-bath) were compared between infected freshwater bathed treatments, infected saltwater bathed treatments and uninfected treatments using a one way analysis of variance (ANOVA), a Tukey' post-hoc test was used when significant differences were detected. Prior to analysis, Levene's test of equality of variances was used to determine the homogeneity of the data sets. Furthermore, a repeated measures 1-way ANOVA was used to detect differences between U_{crit} tests for each treatment, a Tukey's test was used when significant differences were

detected. Paired samples t-tests were used to determine if there was a significant difference between the pre- and post- bath recovery ratios for each treatment.

4.3 RESULTS

4.3.1 Effect of amoebic gill disease on the swimming performance of Atlantic salmon

The recovery ratio (RR) for uninfected fish were 1.00 ± 0.01 (mean ± SEM), whilst fish with all levels of AGD infection had an average RR of 0.87 ± 0.02, which was significantly less than that of uninfected fish (P = 0.001; Table 4.1). Four out of six infected fish that had less than 20% infected filaments had RR's that fell below 0.95, all fish that had more than 20% infected filaments had RR's below the 0.95 threshold (Figure 4.1). Uninfected fish had significantly higher $U_{crit\ 1}$ (P= 0.018) and $U_{crit\ 2}$ than infected fish (P = 0.001, Table 4.1). When comparing within treatments, there was no significant difference between the $U_{crit\ 1}$ and $U_{crit\ 2}$ values of uninfected fish (P = 0.978), however, the $U_{crit\ 2}$ values for infected fish were significantly lower than that of the $U_{crit\ 1}$ values (P = 0.001).

No significant differences were found between infected and uninfected groups for $MO_{2\ rout}$, $MO_{2\ max}$ and metabolic scope (Table 4.1). There was, however, a significant difference (P = 0.04) in EPOC between infected fish and uninfected fish. Furthermore, infected fish took significantly longer to return to baseline routine metabolic rate levels (Table 4.1).

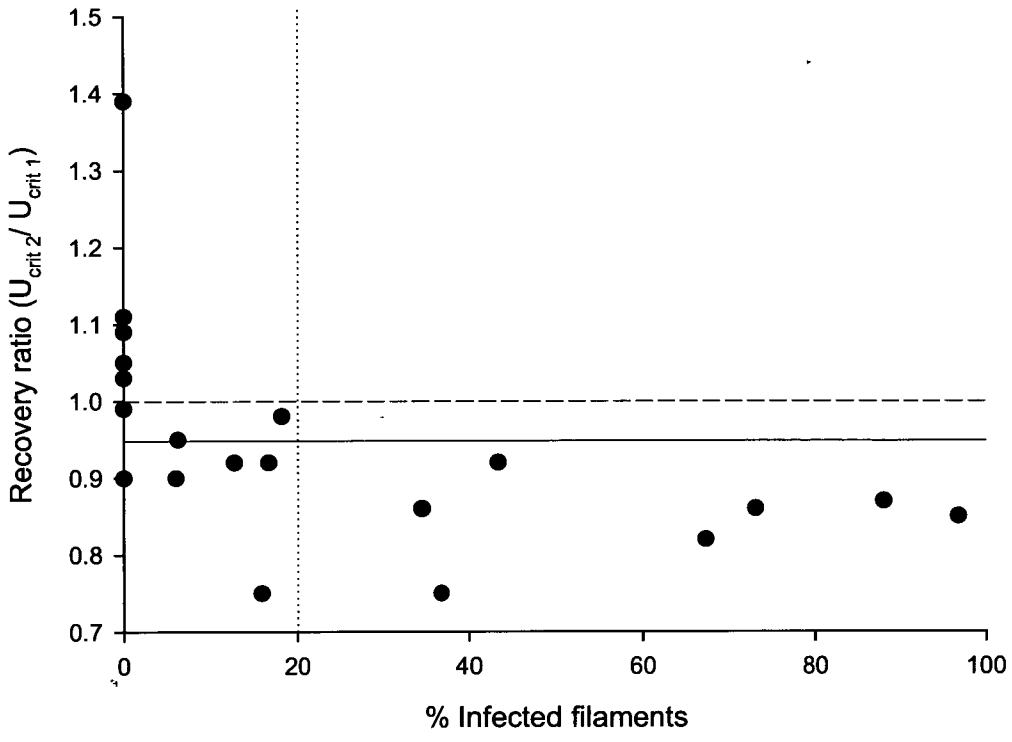

Figure 4.2.

Scatterplot of recovery ratio ($U_{crit\ 2}$/ $U_{crit\ 1}$) versus the percentage of infected filaments. Solid line indicate a recovery ratio of 0.95, horizontal dotted line indicates a recovery ratio of 1. The vertical dotted line indicates 20% infected filaments. A total of 20 fish were used in the experiment, 6 uninfected controls and 14 infected fish with a range of levels of infection.

Table 4.1.

Critical swimming speed tests ($U_{crit\ 1}$ and $U_{crit\ 2}$) measured in body lengths per second ($BL.s^{-1}$) and recovery ratio (RR) of Atlantic salmon showing clinical signs of amoebic gill disease (AGD). An asterix (*) indicates a significant difference between $U_{crit\ 1}$ and $U_{crit\ 2}$ tests for each treatment (infected and uninfected), # indicates significant difference between infected and uninfected treatments for each variable. Excess post-exercise oxygen consumption rate (EPOC) is measured in mg $O_2\ kg^{-1}$ (Lee et al 2003), routine and maximum metabolic rates and metabolic scope are given in figures of $\mu m\ O_2.g^{-1}.h^{-1}$. Six uninfected controls and 14 infected fish were used in the experiment.

	Infected	Uninfected	P
$U_{crit\ 1}$	$2.58 \pm 0.16^{\#}$ *	3.11 ± 0.08	0.018
$U_{crit\ 2}$	$2.26 \pm 0.16^{\#}$	3.11 ± 0.07	0.001
RR	$1.00 \pm 0.01^{\#}$	0.87 ± 0.02	0.010
EPOC	$259.60 \pm 56.47^{\#}$	414.17 ± 55.24	0.040
Time to recovery	$183 \pm 36.39^{\#}$	282.35 ± 17.65	0.011
$MO_{2\ rout}$	4.41 ± 0.49	3.65 ± 0.32	0.195
$MO_{2\ max}$	12.28 ± 1.13	12.04 ± 0.50	0.828
Scope	7.87 ± 2.65	8.38 ± 3.61	0.632

4.3.2 Effect of freshwater bathing on the swimming performance of Atlantic salmon with AGD

Histological examination of the gills revealed that lesions were still present post-bath, however, no amoebae were associated with the lesions (Figure 4.2). Moreover, the lesions that were observed showed high degrees of fragmentation, with obvious breaks between the hyperplastic tissue and the lamellae.

All data sets passed the assumption of Levene's test of equal variances. There was no significant difference in RR_1 pre-bath between the freshwater (FWB) and saltwater bathed (SWB) infected fish, however RR_1 of uninfected fish was significantly higher pre-bath than for the other two treatments ($P = 0.01$) (Table 4.2). The post-bath recovery ratio (RR_2) of FWB fish was not significantly different to uninfected controls (Table 4.2). However, SWB fish had RR_2 that was significantly lower than those of both the FWB fish and the uninfected controls ($P = 0.001$). When comparing within treatments, only the FWB fish had a significant change in RR ($P=0.001$), there was no significant change in RR for either the controls ($P = 0.243$) or the SWB fish ($P = 0.200$).

Comparing individual U_{crit} tests between treatments showed that the $U_{crit\,1}$ of uninfected fish was significantly higher than that of the FWB fish ($P = 0.001$), no differences were found between the FWB fish and the SWB fish, this pattern was repeated for $U_{crit\,2}$ ($P = 0.001$) (Table 4.2). Uninfected treatments had a $U_{crit\,3}$ that

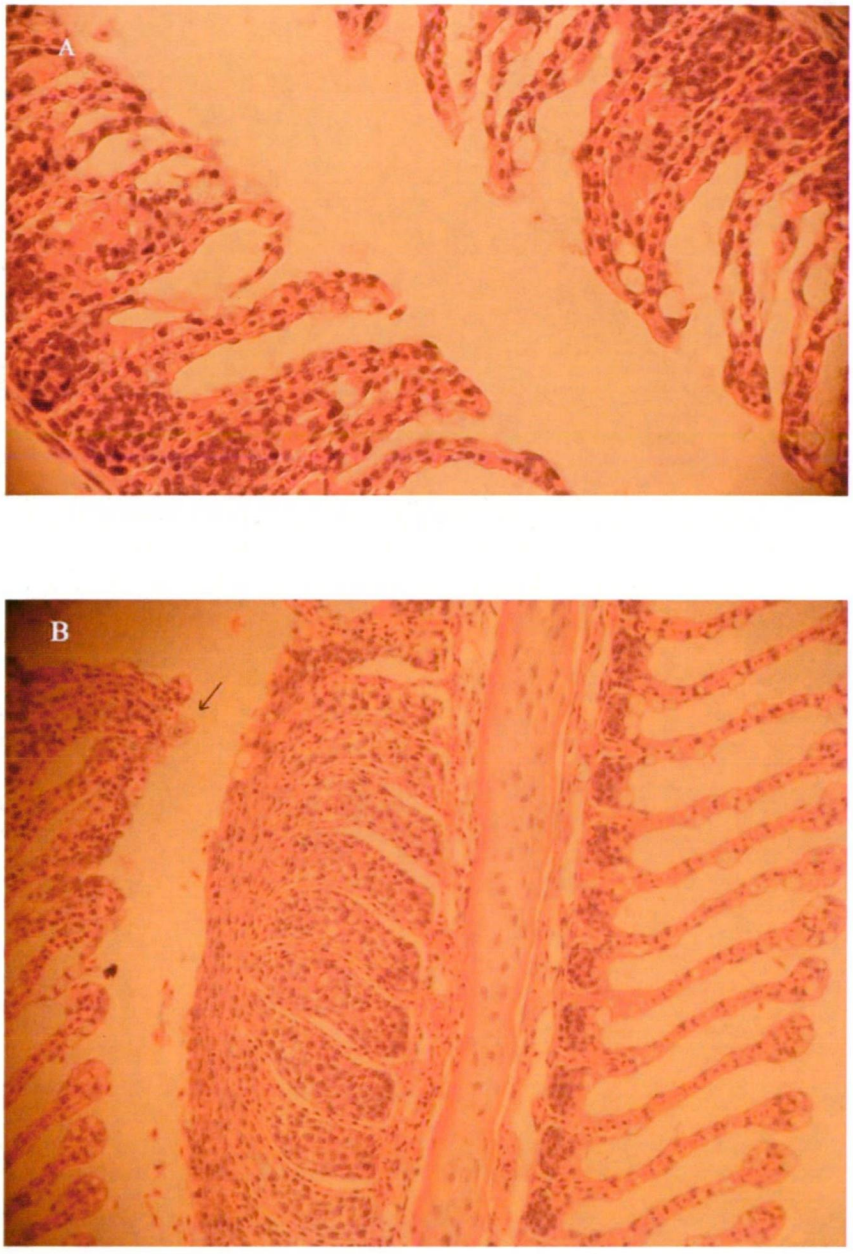

Figure 4.3.

(A) Histology image (400 x magnification) of the gills of an Atlantic salmon (*S. salar*) Affected by AGD 24 hrs post freshwater bath. There is a high degree of fragmentation, and no amoebae are associated with the lesion. Figure 4.2 (B). Typical lesion associated AGD, arrow points to an amoeba (200 x magnification).

was significantly higher than that of the FWB fish, which in turn was significantly higher than that of the SWB fish (P = 0.001), this was also the case for $U_{crit\ 4}$ (P= 0.001).

There was no significant differences between the U_{crit} tests ($U_{crit\ 1-4}$) pre- and post bath for the uninfected controls (P = 0.339) (Table 4.2). The first U_{crit} test was significantly higher than $U_{crit\ 2}$ for the infected FWB fish (P =0.001). Furthermore $U_{crit\ 3}$ and $U_{crit\ 4}$ were significantly higher than $U_{crit\ 2}$, there was no significant difference between $U_{crit\ 4}$ and $U_{crit\ 1}$. The first U_{crit} test of the SWB fish was significantly higher than that of $U_{crit\ 3}$ and $U_{crit\ 4}$ (P = 0.001), there was no difference between $U_{crit\ 1}$ and $U_{crit\ 2}$, nor was there any difference between $U_{crit\ 2}$, $U_{crit\ 3}$ and $U_{crit\ 4}$.

Table 4.2.

Critical swimming speed tests ($U_{crit\ 1}$, $U_{crit\ 2}$ $U_{crit\ 3}$, $U_{crit\ 4}$) measured in body lengths per second ($BL.s^{-1}$) and recovery ratio (RR) of Atlantic salmon showing clinical signs of amoebic gill disease (AGD). Lower case letters (a,b,c) indicates a significant difference between treatments. Upper case letters (X,Y,Z) indicate significant difference between U_{crit} tests for each treatment (infected freshwater bathed, infected saltwater bathed and uninfected. Asterix (*) indicates significant difference between RR_1 and RR_2 for each treatment. Six uninfected controls were used in this experiment, 10 fish were affected by AGD and were freshwater bathed, 5 fish were affected by AGD and saltwater bathed.

	Treatment		
	Control	FW bathed	SW bathed
$U_{crit\ 1}$	3.10 ± 0.04^{a}	1.48 ± 0.11^{bXY}	1.47 ± 0.07^{bX}
$U_{crit\ 2}$	2.99 ± 0.06^{a}	1.01 ± 0.11^{bX}	0.84 ± 0.15^{bXY}
RR	0.96 ± 0.01^{a}	0.67 ± 0.06^{b}*	0.56 ± 0.12^{b}
$U_{crit\ 3}$	3.04 ± 0.07^{a}	2.02 ± 0.12^{bZ}	0.79 ± 0.08^{cY}
$U_{crit\ 4}$	3.03 ± 0.07^{a}	1.94 ± 0.11^{bYZ}	0.42 ± 0.05^{cY}
RR_2	1.00 ± 0.02^{a}	0.97 ± 0.04^{a}	0.46 ± 0.11^{b}

4.4 DISCUSSION

The major conclusions from the first experiment are that AGD significantly affects the critical swimming speed of Atlantic salmon and their subsequent ability to recover and perform a second swimming performance test. Four out of six fish with relatively minor infections (< 20% infected filaments), had recovery ratios that fell below 0.95, suggesting that even relatively minor infections can have a significant impact on the fish's ability to perform a repeat swim performance test. The second experiment showed that freshwater bathing significantly increases the swimming performance of Atlantic salmon that are affected by AGD, and the post-bathing increase in swimming performance may be related to the direct removal of amoebae and hyperplastic tissue from the gill surface. Bathing in freshwater has been shown to reduce viable amoeba counts from 74% to 41% of total amoeba present on the gill in fish that have 65% infected filaments (Roberts and Powell, 2003). There was a significant increase in U_{crit} and RR 48 h post-bath in the FWB fish, that this was not found in the SWB fish suggests that the effect was due to the freshwater bathing rather than a handling artefact. Recovery ratios of FWB fish increased from a pre-bath level of 0.67 to post-bath level of 0.97, which was not significantly different from that of the uninfected fish. Interestingly, histological examination of freshwater bathed gills indicated that lesions were still present on the gill filaments, however, they were fragmented and appeared to be coming away from the gill epithelium, suggesting that the freshwater bathing process enables the

fish to shed the gill lesion-associated hyperplastic tissue, possibly as a result of osmotic pressure and changes to the mucous layer (Roberts and Powell, 2003). It is unlikely that the use of clove oil as an anaesthetic had any adverse effect on the amoebae, upon completion of the experiment gill histological examination revealed the presence of lesions associated with amoebae and the presence of the amoebae themselves in fish that had not been freshwater bathed, suggesting that the clove oil had a minimal impact on the viability of the amoebae. Amoebic gill disease had a significant impact on the excess post-exercise oxygen cost (EPOC) and time to recovery in fish with AGD, however, there was no significant impact on $MO_{2\ rout}$, $MO_{2\ max}$ or metabolic scope.

Of the few studies that have published results on the effect of a disease on U_{crit} and recovery ratios, most have found a significant reduction in the fishes ability to perform a repeat swim test (Swanson et al, 2002; Tierney and Farrell, 2004; Tierney et al 2005; Wagner et al., 2005). A reduction in swimming performance is not site specific with regards to the location of the infection, instead it appears that a number of diseases affecting a number of different organs can illicit a reduction in swimming performance. In the extensive study published by Tierney and Farrell (2004), the effect of several levels of disease (i.e. *Ichthyophonus* spp. heart infection, *Saprolegnia* spp. gill infection) and injury (i.e. skin wounds) on the repeat swimming performance of sockeye salmon (*O. nerka*) were examined, and it was shown that diseased fish had significantly impaired repeat swim performance ability, particularly at high disease loading levels. Adult sockeye salmon had an 8%

reduction in recovery ratios when exposed to a controlled infection of the myxosporean kidney parasite *Parvicapsula minibicornis* (Wagner et al., 2005), sea lice (*L. salmonis*) have also been shown to have a significant impact on the critical swimming speeds of adult Atlantic salmon (Swanson et al., 2002). In contrast, rainbow trout (*O. mykis*) with an induced subclinical *Listonella anguillarum* infection had recovery ratios that were above the levels of those observed in controls (Tierney et al., 2005) suggesting that response to disease with regards to swimming performance may be in some instances, disease or pathogen specific. Swimming performance tests have been shown numerous times to be an effective non-lethal technique to assess the impact of disease on aquatic organisms, the current study adds to this assertion.

The failure of fish with a low level of infection (<20% infected lesions) to successfully complete a second U_{crit} test equivalent to the first U_{crit} test is of particular interest. Fish may opt to swim at different U_{crit} levels depending on environmental or physiological conditions (Jain and Farrell, 2003; Tierney et al., 2005). It is possible that whilst fish with low infection levels managed to maintain $U_{crit\ 1}$ levels equivalent to those of fish with no signs of AGD, it came at an increased physiological cost through a greater use of anaerobic exercise, which would lead to a greater accumulation of post-exercise metabolites such as lactate and cortisol (Jain and Farrell, 2003; Tierney et al., 2005), this would in turn hinder the fish's ability to perform a second U_{crit} test (Tierney et al., 2005). Salmon performing U_{crit} tests typically change from primarily aerobic locomotion to

anaerobic locomotion at speeds approaching 70 – 80% of U_{crit} (Burgetz et al., 1998). As the use of anaerobic metabolism increases, so does plasma lactate levels, to a point at which plasma lactate levels become the limiting factor in repeat swim performance tests (Farrell et al., 1998). It has been stipulated that once a fish has reached a plasma lactate level of above approximately 10 μmol L^{-1} the ability of the fish to perform a repeat swim test becomes severely impaired, even at low swimming speeds (Farrell et al., 1998; Stevens and Black, 1966). Indeed plasma lactate levels were above 7 μmol L^{-1} an hour after exhaustive exercise and failed to return to baseline levels after 6 h in AGD affected salmon with less than 20% infected filaments (Powell et al., 2003). An increase in plasma lactate levels indicates that the energy requirements of the muscles during exhaustive exercise is not being met by aerobic metabolism, and instead must be met by stores of high energy phosphates and the anaerobic metabolism of glycogen, resulting in the formation of lactate (Jobling, 1994). Whilst it has been shown that oxygen diffusion in the gills of Atlantic salmon with AGD is not a limiting factor during routine oxygen consumption, it is not known whether oxygen uptake becomes limiting during times of high oxygen demand (Powell et al., 2000). Furthermore, AGD causes profound cardiorespiratory effects that may also affect the swimming performance of fish. Atlantic salmon with AGD with less than 20% infected filaments had a 35% reduction in cardiac output, associated with a reduction in cardiac stroke volume and an increase in vascular resistance, a condition that is restored to normal levels after a freshwater bath (Leef et al., 2005a; Leef et al., 2007). Maximum cardiac performance and swimming performance are intrinsically

linked, with a reduction in cardiac output resulting in a reduction in swimming performance (see review by Farrell, 2002). The restoration of normal vascular resistance and the direct removal of hyperplastic gill tissue and amoebae after a freshwater bath may account for some of the recovery in swimming performance observed post-freshwater bath for salmon in the second experiment (Powell et al., 2002b). Furthermore, the amoebae themselves may be directly impacting on swimming performance, the cause of the hypertension and reduced cardiac output observed in previous experiments has not been directly identified (Powell et al., 2002; Adams and Nowak, 2003; Leef et al. 2005a), and it is not known whether it is a function of the restrictive nature of the lesions on the gill filaments, or an effect of an exotoxin produced by the amoebae. Gill histological samples taken post-bath in the current trial as well as previous trials (Roberts and Powell, 2003) have revealed the presence of lesions, albeit fragmented and coming away from the epithelium, but the absence of amoebae, giving some evidence to support this theory.

Excess post-exercise oxygen consumption increased significantly with infection, with infected fish having an EPOC that was approximately double that of uninfected fish. Furthermore, infected fish took longer to recover to routine levels than that of uninfected fish. The recovery times for uninfected fish compare favorably to those reported for adult sockeye salmon (*O. nerka*) (>4 h) following a U_{crit} test at 18°C (Lee et al., 2003), similarly a 4 - 6 h recovery time has been reported for juvenile rainbow trout exhausted by either U_{crit} swimming or by chasing (Brett, 1964; Scarabello et al., 1991; 1992). The excess post-exercise

oxygen consumption levels in the current trial are similar to those measured for sockeye salmon following a U_{crit} measurement at 18°C over a 45 minute period (Lee et al., 2003). The results suggest that Atlantic salmon with AGD require more oxygen post-exercise and they require it for a longer period of time, possibly due to a reduced ability to uptake oxygen during periods of exhaustive exercise leading to an increased use of anaerobic metabolism. As previously mentioned, Atlantic salmon with AGD have been shown to have highly elevated plasma lactate levels post-confinement, elevated plasma lactate levels have been shown to correlate to inferior recovery in rainbow trout (*O. mykiss*) (Jain, 1999 cited in Lee, 2003). All research to date has focused on blood gas parameters of resting Atlantic salmon with AGD, however, it is not known whether the presence of the lesions impairs the ability of the salmon to uptake oxygen during periods of high activity (Powell et al., 2004). This has profound implications for the aquaculture industry, as it suggests that fish with AGD may require more oxygen over a longer period of time, particularly during times when physical exertion is at its peak, such as cage movements and bathing operations. Atlantic salmon in Tasmania are often farmed in areas of high water movement, and are often subjected to severe handling episodes such as those that occur during freshwater bathing (the common treatment for AGD in Tasmania). Furthermore, AGD outbreaks often occur during periods of warm water when oxygen availability is at its lowest. The results suggest that Atlantic salmon with AGD may have a reduced capacity to recover from periods of strenuous exercise, and that freshwater bathing may go some way as to alleviating the detrimental effects that AGD has on swimming performance.

CHAPTER 5 The effect of AGD on protein synthesis rates.

5.1 INTRODUCTION

The gill is the primary organ for respiration, as well as playing an important role in nitrogenous excretion and maintaining ionic and osmotic balance (Mommsen, 1984). The gill is also a highly active metabolically, with ion transport and protein synthesis accounting for a majority of the energy consuming processes (Lyndon and Houlihan, 1998). Whilst a significant amount of research exists on the physiology of the gill in apparently healthy fish under ideal environmental conditions, only a limited amount of research has examined the effect of adverse environmental conditions on fish gill physiology (Lyndon and Houlihan, 1998, Powell, 2007). Furthermore, only a handful of studies have examined the effect of environmental influences on protein synthesis and turnover (see review Lyndon and Houlihan, 1998, Carter and Houlihan, 2001), no research has been conductedexamining the effect of disease on protein synthesis rates in the gills of affected fish. This is of particular interest as a number of proliferative gill diseases result in hyperplasia of

pavement cells, chloride cells or mucous cells, thus potentially increasing protein synthesis rates at the organ level.

Gill protein synthesis rates are significantly influenced by external factors, particularly potentially harmful agents such as pollutants and heavy metals (Hogstrand et al, 1994, Wilson et al., 1996). There was a pronounced decrease in synthesis rates in gills of rainbow trout exposed to zinc (Hogstrand et al., 1994), *Cyprinus carpio* gills exposed to nickel showed a similar trend with a decrease in protein levels within the gill (Sreedevi et al., 1992), suggesting an inhibitory effect on synthesis and an increase in catabolism (Lyndon and Houlihan, 1998). In contrast rainbow trout *Oncorhynchus mykiss* gills exposed to a sublethal acid (pH > 5.5) aluminium environment had increased protein synthesis rates 32 days after exposure, after which protein growth levels decreased to that of the controls suggesting an acclimation to the damage caused by the exposure (Wilson et al., 1996). It is thought that epithelial cell turnover, chloride cell turnover and mucus production contribute to the majority of protein turnover in the gills (Lyndon and Houlihan, 1998).

Epithelial cell proliferation and mucus production are often associated with gill diseases, indeed it is one of the defining characteristics of Atlantic salmon *Salmo salar* gills affected by amoebic gill disease Amoebic gill disease (AGD). AGD predominantly affects sea-caged salmonids and is the most significant health issue affecting Atlantic salmon production in Tasmania (Munday et al., 1990; Munday et al., 2001; Nowak et al. 2002). The putative agent is *Neoparamoeba perurans* (Young et al, 2007), the disease manifests as a gill infection and is characterized by

multifocal hyperplasic lesions caused by the fusion of lamellae, hyperplasia of the filamental epithelium, accumulation of branchial mucous cells and cellular debris (Munday et al., 1990; Nowak & Munday, 1994).

Recent work has suggested that there is a significant increase in oxygen consumption in Atlantic salmon affected by AGD (Jones et al., unpublished), however the proportion of this increase that is associated with epithelial cell proliferation and mucous production is not known. Protein synthesis is energetically expensive, 23 mmol O_2 is needed to synthesize 1 g of protein (Smith and Houlihan, 1995). Therefore, whilst it is unlikely that any increase in protein synthesis in the gills will result in an increase in oxygen consumption at an organism level, it is of interest to determine the extent of any increase in oxygen consumption at the organ level (Lyndon and Houlihan, 1998).

Information on the effect of disease on tissue protein turnover rates is limited at best, and to date no work on the effects of proliferative gill diseases on protein synthesis rates has been performed. Therefore the aim of this study was to examine the effect that AGD has on the protein synthesis rates in gill tissue of affected Atlantic salmon. Furthermore, one of the main aims of this study was to determine whether the previously observed increase in epithelial cell proliferation and mucous production was associated with an increase in protein synthesis, and therefore energy consumption. Protein synthesis rates of the white muscle tissue was also examined, primarily as a reference tissue to which gill synthesis rates

could be compared to determine whether AGD was affecting the synthesis rates of white muscle tissue and thus growth.

5.2 MATERIALS AND METHODS

Atlantic salmon smolts with a mean (± SEM) mass of 86.1 ± 4.1 g and a mean fork length of 20.5 ± 1.2 cm were obtained from a freshwater commercial hatchery in Tasmania (Saltas, Australia) and acclimated over two weeks to seawater (35 ‰, 1 µm filtered) at 15°C (± 1°C) in a rectangular fibreglass Rathbun tank (4000 L).

After acclimation experimental fish were housed in a temperature-controlled room (15 ± 0.5°C) that contained four individual 400 L recirculation systems. Each system consisted of a 200 L conical bottom tank and a 200 L sump, which contained a biofilter, and mesh to remove solids and catch uneaten feed. Fifty-six (14 per tank) fish were removed from the seawater acclimation holding tank and placed into the recirculation systems. Water quality was measured daily throughout the experiment, dissolved oxygen remained above 95% saturation, total ammonia levels peaked at 2 mg L^{-1} five days after transfer from the holding tank and was below 0.5 mg L^{-1} thereafter.

5.2.1 Neoparamoeba spp challenge

Neoparamoeba spp were isolated using the techniques previously described (Morrison et al. 2004; Howard and Carson, 1994; Powell and Clark 2003). Briefly, gills of post-mortem Atlantic salmon that had been infected via cohabitation in an experimental challenge AGD tank were excised and placed into a container with 0.2 µm filtered seawater. Individual gill arches were separated and placed into a 50 ml centrifuge container with distilled water and gently agitated for 2 min. The

preparation was centrifuged at 4000 g for 5 min and the supernatant was discarded. The pellet was re-suspended in 0.2 μm filtered seawater, agitated and the liquid poured into petri dishes (approx 20 ml^{-1} dish) while being careful not to pour out the gill debris. The process was repeated 4 times, the petri dishes were then incubated for 1 h at 18°C. After 1 h, the liquid in the petri dishes was decanted and placed onto additional dishes and after 1 h, all petri dishes were rinsed and filled with 0.2 μm filtered seawater and incubated for 24 h at 18°C. Amoeba were harvested by decanting the filtered seawater off the petri dishes, 1 mL of trypsin-EDTA (0.05% trypsin) (Invitrogen) was added. The dishes were gently tapped against the workbench in order to dislodge the amoeba. The supernatant was removed and placed into centrifuge tubes and spun at 4000 g for 5 min, the supernatant was drawn off and the pellet resuspended in 0.2 μm filtered seawater. Amoeba numbers were counted using a haemocytometer. Amoeba were introduced directly into two of the four recirculation systems to give a final concentration of 400 cells L^{-1}.

5.2.2 Routine metabolic rate

Six infected and six uninfected fish were used for metabolic rate and protein synthesis determination, three from each of the two infection tanks and three from each of the two control tanks. A further nine fish were sampled from the uninfected tanks to confirm the time course of phenylalanine incorporation, however metabolic rates were not measured for these fish. Five fish from each of the infected tanks were lethally sampled for gill histological analysis in order to assess levels of infection (see below). Routine metabolic rate measurements were conducted in a

2130 mL black acrylic box, food was withheld for 24 h prior to metabolic rate measurements to ensure that fish were in a post-absorptive state (Jones et al., 2007).. Twelve hours prior to the Mo_2 measurements fish were anaethetised using a 0.005% solution of clove oil, an assessment was made of the number of visible lesions on the hemibranchs of the gills (Adams and Nowak, 2003), after which they were immediately transferred into the respirometry boxes Water was supplied to the respirometry boxes via a 90 L recirculation system, water temperature was maintained at 15°C ± 0.2°C (± range), oxygen was maintained at > 97% air saturation by an air stone in the header tank.

Prior to all metabolic rate measurements, a thermostatically controlled oxygen electrode (1302 electrode, Strathkelvin instruments Ltd Glasgow, UK) was calibrated using a 2% $NaSO_3$ (zero) solution and air saturated seawater (155 mm Hg). The electrode was attached to a Strathkelvin instruments model 782 O_2 meter. To determine routine metabolic rate ($Mo_{2\ rout}$), water and airflow to the boxes was stopped and the box sealed. A 3 mL sample of water was removed from the box and injected into the oxygen electrode. After 10 min a final water sample was taken and the oxygen content re-measured (Powell et al., 2005; Jones et al., 2007).

Metabolic rate was calculated using the following formula:

$$Mo_2 = \frac{((PO_{2i} - PO_{2e}) * \alpha) * (V - M)}{T * M} \qquad [1]$$

where PO_{2i} and PO_{2e} are the initial and final oxygen tensions respectively (mm Hg), α is the molar O_2 solubility in water ($\mu M\ O_2.L^{-1}$ mm Hg^{-1}), V is the

respirometer box volume (L), T is the time between the initial and the final oxygen measurements (s) and M is the mass (g) of the fish (Cameron, 1986; Cech, 1990).

5.2.3 Protein synthesis

Protein synthesis was determined by using the flooding-dose method that has been previously described (Garlick et al., 1980; Houlihan et al 1986; Carter et al., 1993). Oxygen consumption rates were measured for each fish prior to protein synthesis measurements. Fish were then anaesthetised (100 mg L^{-1} benzocaine), weighed and injected into the caudal vein with ^{3}H phenylalanine at a rate of 1 ml per 100 g of body weight. Injection solution comprised of 150 mmol L^{-1} phenylalanine and L-[2,6 – ^{3}H] phenylalanine (Amersham Pharmacia Biotech, NSW, Australia) in saltwater Cortland's solution. Specific activity of the injection solution was 1123 ± 118 dpm $nmol^{-1}$ phenylalanine. After injection the fish were returned to 50 L aerated containers (15°C) until sampled, time between injection and sampling ranged between 20 – 34 min. An additional nine fish were sampled (gill and white muscle tissue) at 20 (19 – 22 min), 40 (36 – 46 min) and 60 min (60 -63 min) post injection with 3 fish sampled per time interval to investigate the time course of phenylalanine incorporation to validate that the free pool concentrations were stable and phenylalanine incorporation was linear. After incubation fish were removed from the containers and euthanised in benzocaine (400 mg L^{-1}, benzocaine). Samples of white muscle were taken from above the lateral line, below the dorsal fin and immediately frozen in liquid nitrogen. Furthermore, the entire gill basket

was excised, individual arches dissected out and frozen in liquid nitrogen. The bound and free-pool data from the control fish for both the white muscle and gill tissue were also added to the validation graph in order to increase the accuracy of the regression.

Analysis of samples to measure protein bound and free pool phenylalanine specific radioactivity was conducted using methods previously described (Houlihan et al 1986; Carter et al., 1993). Briefly, approximately 100 mg of frozen tissue was homogenised in ice cold 0.2M perchloric acid (PCA) and centrifuged, the PCA in the supernatant was precipitated with tripotassium citrate. The solution was then centrifuged, leaving the free pool in solution. Protein in the PCA extracted pellet was resuspended in 0.3M NaOH, samples where taken for analysis of protein and [^{3}H]phenylalaine content. Protein estimations were determined using a modification of the folin-phenol method (Lowry et al., 1951), [^{3}H]phenylalaine content was analysed by liquid scintillation counting. A further sample was acidified with PCA and centrifuged, the supernatant was analysed for RNA concentration in the digested tissue the using dual wavelength absorbance method (Ashford and Pain, 1985). The pellet was washed three times with PCA then hydrolysed in 6M HCl for 18hrs at 90°C. The acid was evaporated off using a rotary evaporator, after all the acid had been evaporated the residues were resuspended in sodium citrate buffer. Phenylalanine in the suspension and in the free pool samples were converted to β-phenylalanine using L-tyrosine decarboxylase, extracted with heptane and analysed using the ninhydrin reaction. The [^{3}H]phenylalanine was measured using liquid

scintillation counting. Fractional rates of protein synthesis (k_s) were calculated using the following formula;

$$k_s\ (\%\ d^{-1}) = (S_b/S_a) \times (1440/t) \times 100$$

(Garlick et al (1980)) where S_b and S_a are the protein bound and free phenylalanine specific radioactivities after an incorporation time (t min).,. RNA was expressed as the capacity for protein synthesis (C_s: mg RNA. g protein^{-1}) and as RNA activity (k_{RNA}, k_s. g^{-1} RNA. d^{-1}) (Sugden and Fuller, 1991).

Absolute protein synthesis rates A_s (mg.h^{-1}) in the gill were calculated using the following formula;

$$\frac{(\%protein \times gill\ weight(g)) \times k_s}{24} \qquad [1]$$

Energetic cost of gill protein synthesis (μM O_2.g of gill tissue .h^{-1});

% protein content of gills x gill weight (g) [2]

[2] x k_s = g protein synthesised. day^{-1} [3]

[3] x 8.3 mM O_2.g protein synthesised = mM O_2.day^{-1} [4]

$$\frac{[4]}{24} \times 1000 = \mu M\ O_2.h^{-1} \qquad [5]$$

$$\frac{[5]}{gillweight(g)} = \mu M\ O_2.g.h^{-1}$$

To calculate the percentage of the $MO_{2\,rout}$ attributed to k_s in the gills the following formula was used;

$$\frac{(MO_{2\,rout}\ (\mu M\ O_2.g.h^{-1})\ x\ \textit{fish weight}(g)\ x\ 24)}{1000} = mM\ O_2\ .day \qquad [6]$$

$$\frac{[4]}{[6]} \times 100 = \%\ of\ MO_{2\,rout}\ attributed\ to\ gill\ k_s$$

5.2.4 Histology

Five fish from each of the infection tanks were lethally sampled on the day of injection for histological analysis (see below). Fish were given a lethal overdose of clove oil (0.05%), gills were excised from the fish and rinsed gently in seawater to remove excess blood before being placed into saltwater Davidson's fixative for histological assessment of AGD severity.

Twenty-four hours after being placed into the Davidson's fixative, the gill basket was transferred into 70% ethanol. The second left anterior arch was removed, dehydrated, embedded in paraffin wax, sectioned at 5 μm and stained with haematoxylin and eosin (H & E). Sections were then viewed at 100 x magnification under a light microscope (Olympus, Hamburg, Germany). The number of filaments exhibiting AGD lesions were counted and expressed as a proportion of the total number of filaments counted (Adams and Nowak, 2001; Parsons et al., 2001). Furthermore, the lesions present were counted and expressed as the total number of lesions per filament. A filament was only counted when the lamellae were of equal

length bilaterally and present to near the tip of the filament, and the central venous sinus was visible for at least two-thirds of the filament (Speare et al., 1997).

5.2.5 Statistics

Statistical analyses were conducted using the statistical package SPSS for Windows (Version 11.5). Weights, lengths, $MO_{2\ rout}$, total RNA concentration, k_{RNA}, k_s, and C_s were analysed between AGD affected and control fish using Student's T-Test. A 1-way ANOVA was used to determine if the free pool phenylalanine incorporation in the white muscle and gill tissue was linear over time. Results are presented as mean ± SEM, data was significantly different if $P < 0.05$.

5.3 RESULTS

There were no significant differences in weight or length between the two treatments, control fish had a mean weight of 88.47 ± 8.84g, whilst fish with AGD had a mean weight of 80.10 ± 11.45g. Visual inspection of the gills of fish with AGD used for protein synthesis analysis indicated heavy infection levels (10-16 hemibranchs affected). Histological analysis of satellite fish taken from the same infection tank showed an average infection level of 59.6 ± 9.3% (mean ± SEM) infected filaments.

5.3.1 Protein synthesis

There was a significant relationship between the bound ^{3}H phenylalanine (dpm.nmol^{-1} phenylalanine) and *in vivo* incubation time for the white muscle tissue, described by the formula $y = 0.0051x + 0.1904$ ($p = 0.0021$, $df = 14$, $r^2 = 0.529$, Figure 5.1a). There was no relationship for free-pool ^{3}H-phenylalanine and time for white muscle tissue ($p = 0.730$, $df = 14$, $r^2 = 0.18$, Figure 5.1b) and the mean specific activity was 1239 ± 151 dpm.mol^{-1} phenylalanine. The free pool phenylalanine specific radioactivity in the white muscle tissue at 60 min post injection was not significantly different from the specific radioactivity at 20 min or 40 min ($P = 0.645$). Similarly, for gills there was a significant relationship between bound ^{3}H phenylalanine and *in vivo* incubation time, $y = 1.9489x - 1.3708$ ($p = 0.001$, $df = 14$, $r^2 = 0.607$, Figure 5.2a). There was no significant relationship between free-pool ^{3}H-phenylalanine ($p = 0.6287$, $df = 14$, $r^2 = 0.005$, Figure 5.2b) and in vivo incubation time for gill tissue, with a mean specific activity of 1896 ± 192 dpm.mol^{-1} phenylalanine. Furthermore, there was no significant difference in free pool phenylalanine specific radioactivity in the gill tissue at 60 min when compared to levels at 40 and 20 min ($P = 0.703$). These data confirmed the white muscle tissue and gill tissue free pools remained flooded over the incubation time and the incorporation rate was constant.

Use of the flooding dose method is well established in salmonids and injection as done in the present study will flood the tissue free pools. Consequently, the pre-

injection free pools were not measured in the present study. However, the stable specific radioactivity in the free pool and the linear incorporation (Figure 5.1 and 5.2) show the tissue was flooded and behaving as such. In addition, the normal free pool phenylalanine concentration for salmon is 0.8 - 1.4 nmol.g^{-1} wet weight (Carter et al., 2000), in the present study the flooding dose elevated this to 44 nmol.g^{-1} wet weight for the gill and 23 nmol.g^{-1} wet weight for the muscle tissue.

There were no significant differences in protein synthesis rates (k_s) between AGD affected fish (2.11 ± 0.34 % d^{-1}) and control fish (2.31 ± 0.88 % d^{-1}) for white muscle (P = 0.82), nor were there any significant differences in RNA concentration (P = 0.76), k_{RNA} (P= 0.46) or C_s (p = 0.144) values (Table 5.1). For gill tissue, fish affected by AGD had a mean k_s of 13.56 ± 2.05 % d^{-1} that was significantly (P = 0.037) higher than the control, 8.05 ± 0.97 % d^{-1} (Table 5.1). There were no significant differences in RNA concentration (P = 0.34), k_{RNA} (P = 0.89) or C_s (P = 0.76) values.

The relationship between C_s and k_s was not significant for white muscle tissue (p = 0.882, df = 10, r^2 = 0.003) or gill tissue (p = 0.329, df = 10, r^2 = 0.106) when all treatments were combined. There was a significant positive relationship between k_{RNA} and k_s for the white muscle tissue (p = 0.001, df = 10, r^2 = 0.6505) which could be described by the formula y = 0.4716x + 0.8558. Similarly, a positive relationship between K_{RNA} and k_s (p = 0.15, df = 10, r^2 = 0.491) for gill tissue was

found and could be described by the formula y = 2.3275x + 5.96382, suggesting that ribosomal activity and not the specific RNA concentration was driving protein synthesis.

5.3.2 Oxygen consumption rates

There was no significant difference between the $Mo_{2\ rout}$ of control and AGD affected fish (P = 0.254), with control fish having an average $Mo_{2\ rout}$ of 7.87 ± 1.03 μM $O_2.g^{-1}.h^{-1}$, and AGD affected fish having an average $Mo_{2\ rout}$ of 10.23 ± 1.67 μM $O_2.g^{-1}.h^{-1}$. There were no correlations between whole animal oxygen consumption and the white muscle or gill RNA concentration, C_s, k_{RNA} or k_s values when treatments were analysed individually or combined.

Absolute rates of protein synthesis in gills were 0.806 ± 0.01 $mg.h^{-1}$ for control fish, and 1.13 ± 0.01 $mg.h^{-1}$ for AGD affected fish which was not significantly different from control fish (Table 5.2). Gill protein synthesis for control fish accounted for 2.12 ± 0.43 μM $O_2.g^{-1}.h^{-1}$ or 1.05 ± 0.2 % of the oxygen consumed during $Mo_{2\ rout}$, for AGD affected fish this rose to 3.69 ± 0.63 μM $O_2.g^{-1}.h^{-1}$ or 1.19 ± 0.28 % of the oxygen consumed during $Mo_{2\ rout}$ (Table 5.2), an increase of 1.57 μM $O_2.g^{-1}.h^{-1}$. There was no relationship between absolute rate of protein synthesis (mg protein. day^{-1}) and oxygen consumption (μM $O_2.day^{-1}$) for the control (p = 0.611) or the AGD affected fish (p = 0.4384).

Table 5.1.

Effect of AGD on fractional rates of protein synthesis and RNA (mean ± SEM) concentration in white muscle and gill tissue of Atlantic salmon. Six control and six AGD affected fish were used in this trial.

	Control	AGD affected
White muscle		
k_s (%.day^{-1})	2.31 ± 0.88	2.11 ± 0.34
RNA (μg.mg^{-1})	1.45 ± 0.18	1.55 ± 0.13
C_s (mg RNA.g protein^{-1})	7.21 ± 0.75	9.75 ± 0.76
K_{RNA} (Ks.g^{-1} RNA.d^{-1})	3.63 ± 0.88	2.42 ± 0.48
Gill tissue		
k_s (% day^{-1})	8.05 ± 0.97	13.56 ± 2.05*
C_s (mg RNA.g protein^{-1})	69.21 ± 18.13	78.52 ± 13.00
K_{RNA} (Ks.g^{-1} RNA.d^{-1})	1.79 ± 0.58	2.12 ± 0.55
RNA (μg.mg^{-1})	4.53 ± 1.03	6.33 ± 1.23

C_s is the RNA to protein ratio (mg RNA.g protein $^{-1}$), K_{RNA} is the RNA activity (k_s.g^{-1} RNA.d^{-1}). Asterix indicates a significant difference ($p < 0.05$) between control (n = 6) and AGD affected fish (n = 5).

Table 5.2.

Energetic cost of protein synthesis in the white muscle tissue and the gill tissue of Atlantic salmon affected by AGD. Six control and 6 AGD affected fish were used in this trial.

	Control	AGD affected
% of $Mo_{2\ rout}$ attributed to k_s	33.49 ± 2.55	10.65 ± 2.07
Gill protein synthesised ($mg.h^{-1}$)	0.81 ± 0.01	1.13 ± 0.01
[1]Energetic cost of gill k_s ($\mu M\ O_2.g^{-1}.h^{-1}$)	2.12 ± 0.43	3.69 ± 0.63
% of $Mo_{2\ rout}$ attributed to k_s	1.05 ± 0.20	1.19 ± 0.28

[1] Energetic cost of gill protein synthesis expressed as micromole of oxygen consumed per gram of gill tissue per hour.

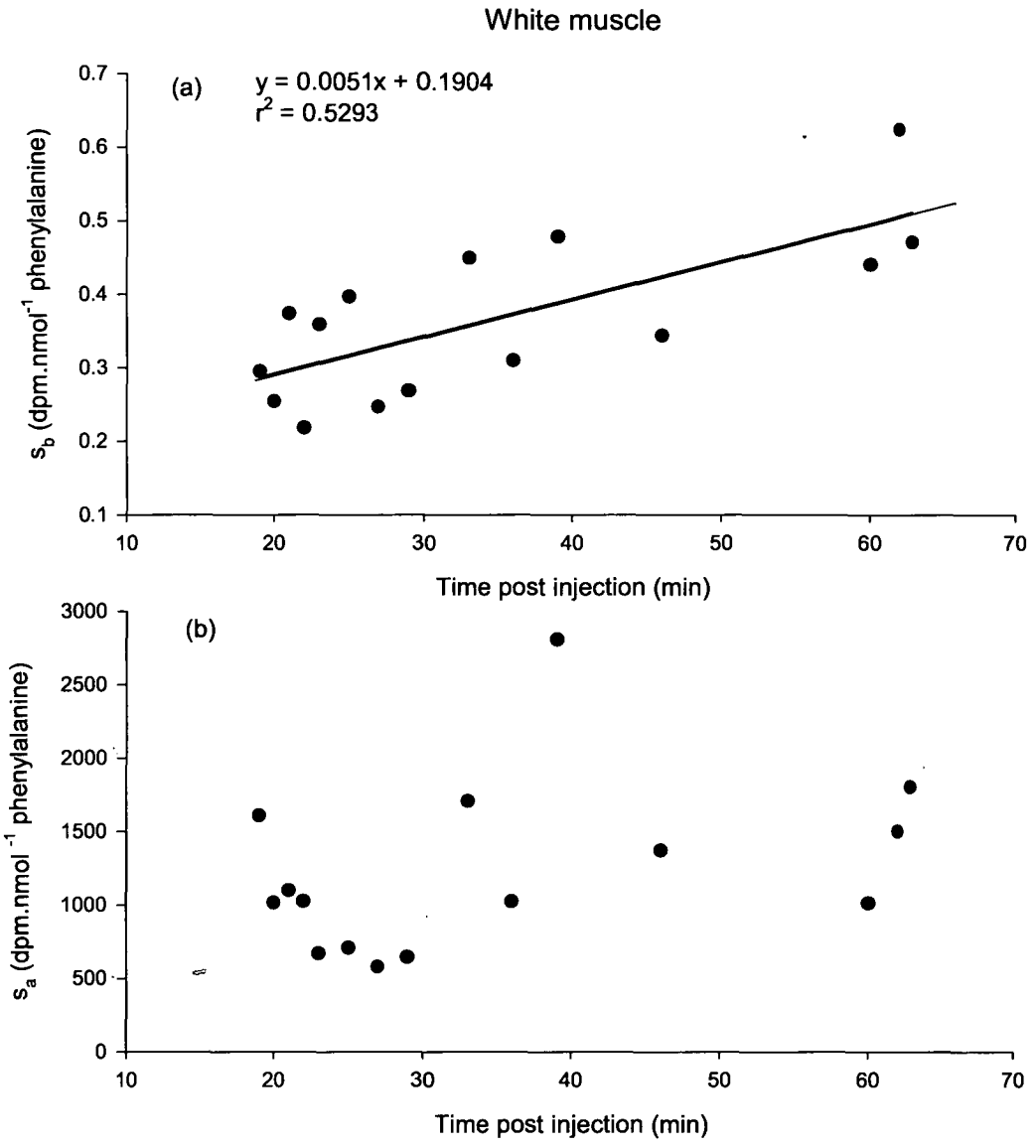

Figure 5.1.

Validation graph (n = 15) examining the relationship between the *in vivo* incubation times and bound ^{3}H phenylalanine (s_b) (a) and free pool (s_a) (b) in the white muscle tissue. A significant relationship (P = 0.0021) exists between invivo incubation time and s_b and can be described by the formula y = 0.0051x + 0.1904. No significant relationship exists between *in vivo* incubation time and s_a.

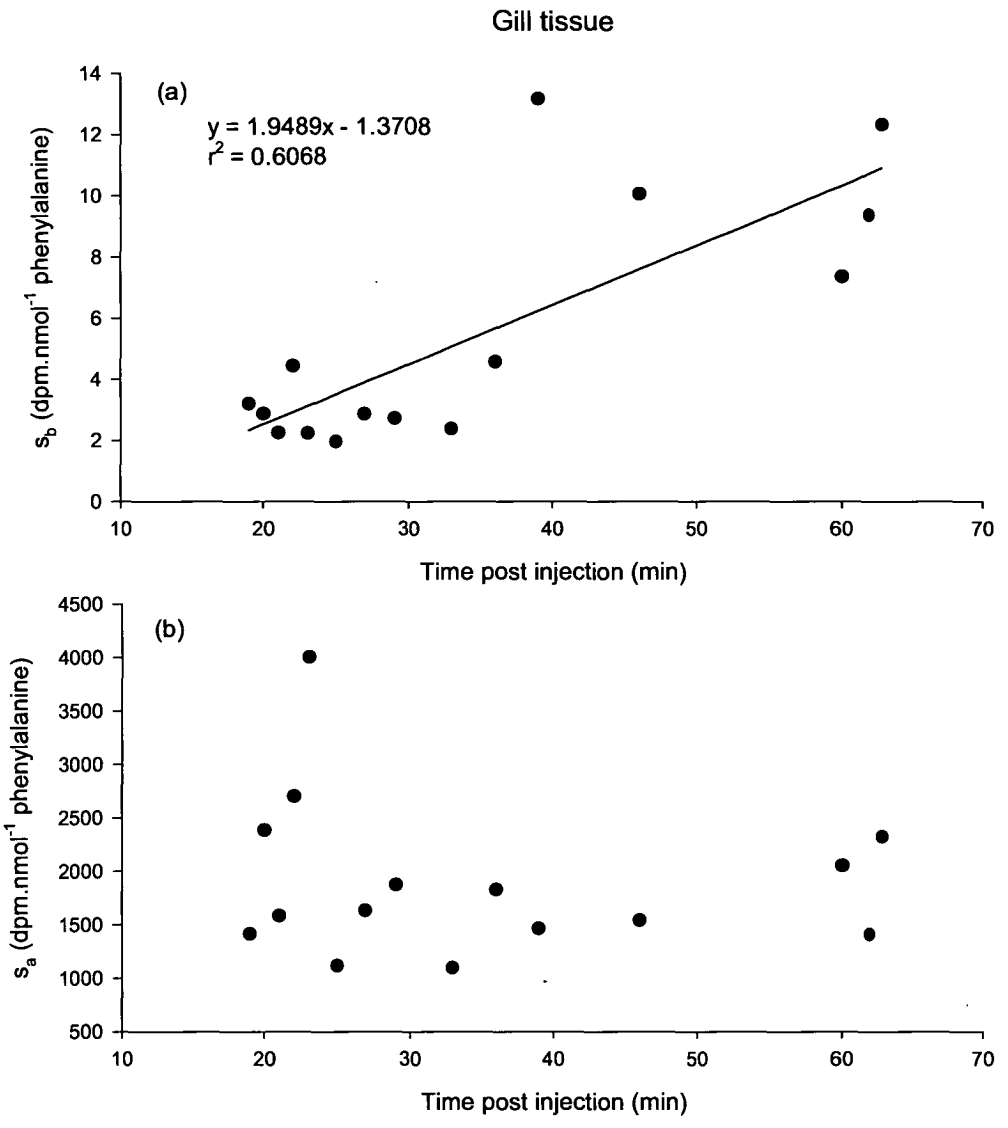

Figure 5.2.

Validation graphs (n = 15) examining the relationship between the *in vivo* incubation times and bound (a) and free pool (b) ^{3}H phenylalanine in the gill tissue. A significant linear relationship exists between *in vivo* incubation time and free – pool ^{3}H-phenylalaline and can be described by the formula y = 1.9489x – 1.3708. No significant relationship exists between *in vivo* incubation time and free – pool ^{3}H-phenylalaline.

5.4 DISCUSSION

This is the first study to examine the effect of a proliferative gill disease on gill protein synthesis. The main hypothesis for this study was that the proliferation of epithelial cells and mucous cells observed in the gills of AGD affected salmon would lead to an appreciable increase in protein synthesis, the results of the current study present evidence that this is indeed the case. Protein synthesis rates for AGD affected salmon were significantly higher than that of the control fish. There was no significant difference between the white muscle synthesis rates in control and infected fish, providing further evidence that the physiological effects of AGD are primarily restricted to the gills (Adams and Nowak, 2001, Adams and Nowak, 2003). The white muscle protein synthesis rates observed in the current trial are similar to those found in other studies on Atlantic salmon, Carter et al (1993) found white muscle synthesis rates in Atlantic salmon at 12.9°C to be 1.4%.d^{-1}, whilst Martin et al (1993) found white muscle synthesis rates in Atlantic salmon to be 2%.d^{-1}. Gill protein synthesis rates of control fish were also similar to those observed for Atlantic salmon in other trials, Noble (1990) found gill synthesis rates of saltwater smolt to be 7.82% d^{-1}, whilst Fauconneau et al (1989) found gill synthesis rates of freshwater parr to be 5.83-5.92%.d^{-1}.

A significant increase in $Mo_{2\ rout}$ equivalent to approximately 3.5 μM $O_2.g^{-1}.h^{-1}$ is associated with AGD (Jones et al. in prep). The results of the current experiment show that a relatively small proportion of the increase is directly attributable to protein synthesis in gill tissue, some of the increase in oxygen

consumption may be attributed to an increase in protein synthesis, although it is unlikely to make up a significant proportion. The energetic costs associated with protein synthesis are usually assumed to be 8.3 mM O_2 per g protein synthesized or 50 mmol ATP per g of protein synthesised (Reeds, 1985; Houlihan et al., 1988, Carter and Houlihan, 2001). Gill tissue only accounts for around 6-14% of the whole body synthesis rates, with whole body synthesis rates accounting for between 11- 42% of total oxygen consumption, so even the proportionately large increase in fractional protein synthesis observed in AGD affected gills in the current study would only represent a relatively small increase in whole body synthesis rates (Lyndon and Houlihan, 1998; Carter and Houlihan, 2001). Indeed this was observed in the current trial, with gill respiration associated with protein synthesis for control fish accounting for only 1.05 ± 0.2 % of the oxygen consumed during $Mo_{2\ rout}$, increasing to 1.19 ± 0.28 % in fish affected by AGD based on theoretical values of cost of protein synthesis. Lyndon and Houlihan (1998) came to a similar conclusion when examining the results of Sreedevi et al (1992), protein synthesis rates in the gills of *Cyprinus carpio* were shown to increase after exposure to sublethal concentrations of nickel. However even a 50% increase in branchial oxygen demand would only account for approximately 3% increase in total oxygen consumption. A similar conclusion was also drawn when examining the gills of rainbow trout *O mykiss* exposed to an acid/aluminium environment (Wilson et al., 1996), suggesting that whilst certain environmental and pathological permutations may lead to a significant increase in protein synthesis rates, and hence oxygen consumption rates on the organ level, the likely effect on whole body oxygen

consumption rates is likely to be minimal when the gills are the primary organ affected (Lyndon and Houlihan, 1998). It should be noted that the calculations of the energetic cost of protein synthesis performed in this study represent the minimum cost of protein synthesis, with the energetic cost based on theoretical values (Carter and Houlihan, 2001, Bowgen et al., 2007; Fraser and Rogers, 2007).

The high fractional protein synthesis rates in gills relative to other organs observed in many studies has been attributed to the large surface area of the gill and the key function that gills play in respiration and ion regulation (Lyndon and Houlihan, 1998; Carter and Houlihan, 2001). It is thought that high turnover of epithelial , chloride and mucous cells may account for a majority of the turnover observed in the gill epithelium (Lyndon and Houlihan, 1998). The increase in protein turnover rates in the gills of juvenile rainbow trout *O. mykiss* exposed to sublethal concentrations of aluminium was associated with an increase in damaged gill tissue, and the subsequent protein degradation and synthesis of new gill tissue (Wilson et al., 1996). In the current study, the gills of AGD affected fish were assessed for lesion development based on the standard scoring system (Adams and Nowak, 2003), with heavy infection levels observed in all fish. Fish taken from the same population that were sampled for protein synthesis analysis had approximately 60% of the gill filaments presenting with lesions, suggesting a very high level of infection within the population. The gills of Atlantic salmon that have been affected by AGD show multifocal hyperplastic lesions, with a significant increase in branchial mucous cells and mucous production (Nowak & Monday, 1994, Powell et

al., 2000, Adams and Nowak, 2003, Roberts and Powell, 2005). After initial attachment of the trophozoites, significant amounts of hypertrophy of the surface epithelial cells and a thickening and eventual fusion of the secondary lamellae occurs (Adams and Nowak, 2003). As lesions develop there is also a localised inflammatory response, with a migration of neutrophils, macrophages and lymphocytes along the central venous sinus, with chloride and epithelial cells sloughed off as the lesion develops (Adams and Nowak, 2001, Adams and Nowak, 2003). Proliferating cell nuclear antigen (PCNA) staining of histological slides of AGD affected gills reveals a significant increase in PCNA positive cells along the basal filamental regions of the gill that corresponds to a significant increase in visible AGD lesions, the undifferentiated epithelial cells that make up the majority of the lesions have shown to be PCNA positive as well (Adams and Nowak, 2003). A concurrent increase in proliferative (PCNA positive) cells and AGD suggests that there is a significant increase in cell proliferation and recruitment in response to infection with *Neoparamoeba spp*, which would, in turn, lead to a significant increase in protein synthesis in order to enable cellular proliferation (Adams and Nowak, 2003). The increase in mucous cells and mucous production associated with AGD would further increasing protein synthesis rates above those observed in controls (Munday et al., 1990; Roberts and Powell, 2005).

Variations in protein synthesis rates in the present study are explained by the variation in k_{RNA}, rather than differences in RNA concentration and Cs. A number of studies have found similar results, and have shown that changes in protein

synthesis were driven by changes in ribosomal activity (McMillan and Houlihan, 1989; Foster et al., 1992; Lyndon et al., 1992). However there was no statistical difference in RNA content or k_{RNA} between AGD affected and control fish, so the underlying causes of the increase in k_s cannot by specified (Wilson et al., 1996).

In summary, this study shows that the undifferentiated epithelial cellular proliferation and increase in mucous production associated with AGD in Atlantic salmon is accompanied by a significant increase in protein synthesis rates. However the energetic cost of the increase in protein turnover is likely to be minimal, suggesting that the observed increase in MO_2 in AGD affected fish is likely to be a combination of an increase in gill protein synthesis and other patho-physiological effects.

Chapter 6 - The effect of replacing dietary fish oil with a stearidonic acid rich oil on metabolic rate and metabolic recovery in seawater Atlantic salmon (*Salmo salar* L.)

6.1 INTRODUCTION

Omega 3 (ω3) series long chain polyunsaturated fatty acids (LC-PUFA), predominantly eicosapentaenoic acid (EPA) (20:5ω3) and docosahexaenoic acid (DHA) (22:6ω3), have a significant influence on exercise performance and metabolic rate of a number of fish, including Atlantic salmon (McKenzie et al., 2001; 2005, Wagner et al., 2004). Whilst the physiological mechanisms behind the effect of fatty acids on exercise performance are not known, a number of studies have highlighted the effect that relative concentrations of tissue FA can have on the cardio-respiratory system (Bell et al., 1991; McKenzie et al.1995; 1997; 1998). Atlantic salmon fed diets with very low ω3/ω6 ratio's developed cardiac lesions and were more susceptible to transport-induced shock syndrome (Bell et al., 1991, 2003, McKenzie, 2001). Increased tissue concentrations of LC-PUFA have also been linked to reduced sarcoplasmic reticulum Ca^{2+} -ATPase activity, which has been found to increase the contractile force of isolated perfused trout hearts (Paige et al., 1996, Ushio et al, 1997, McKenzie, 2001). Fish cardiac performance is intrinsically linked with the aerobic performance because the myocardium is predominantly aerobic muscle. Any change in cardiac performance due to deficiencies in essential FA may translate into a measurable effect on $MO_{2\ rout}$, aerobic capacity (metabolic

scope) and the ability of the fish to recover post intensive exercise (Farrell, 1997, McKenzie 2001).

The need to provide alternatives to fish oil for aquafeeds is recognized and considerable research has investigated alternatives (Rosenlund et al., 2001; Bell et al., 2003; Carter et al., 2003). Whilst some plant oil alternatives have produced promising growth in fish, they generally contain higher concentrations of saturated, monounsaturated and ω6 fatty acids, and do not contain any ω3 LC-PUFA (Beardsell et al., 2002). Although not generally tested, fish grown on a diet deficient in ω3 LC-PUFA may be more susceptible to health problems and are less tolerant to sub-optimal conditions such as hypoxia or crowding stress (McKenzie et al., 1997; McKenzie 2001; Bransden et al., 2003;). High concentrations of ω3 LC-PUFA, particularly EPA and DHA, normally sourced from marine organisms must be provided in the diets of seawater Atlantic salmon because these fatty acids cannot be synthesised in sufficient quantities to meet requirements (Sargent et al., 2002).
Oil from the plant source *Echium plantagineum* L., Boraginaceae, is a potential replacement for fish oil in aquafeeds because it supplies a high amount of stearidonic acid (SDA; 18:4ω3) a precursor of EPA that could potentially avoid the enzymatic bottleneck for the desaturation of linolenic acid (Bell et al., 2006 Tocher et al., 2006; Miller et al. 2007). Earlier studies with Atlantic cod, *Gadus morhua,* and Arctic charr, *Salvelinus alpinus,* showed fatty acid conversion of SDA through to eicosatetraenoic acid (ETA; 20:4ω3) but not through to EPA and DHA (Bell et al., 2006; Tocher et al., 2006). Recent work by Miller et al (2007) has shown that

freshwater Atlantic salmon parr can maintain ω3 LC-PUFA concentration in muscle tissue when fed a Echium oil. However, whether this ability exists in the saltwater phase of the Atlantic salmon life cycle was unknown when the current experiment was completed. The impact of providing a diet rich in SDA but deficient in ω3 LC-PUFA on the metabolic rate and exercise physiology of Atlantic salmon smolt is not known but is of vital importance if the technology is ever to be commercialised.

In the present study, we aimed to test the hypothesis that feeding SDA to Atlantic salmon smolts would not affect routine metabolic rate ($MO_{2\ rout}$), maximum metabolic rate ($MO_{2\ max}$) and metabolic scope, or the ability to recover after a bout of exhaustive exercise, measured as excess post exercise oxygen consumption (EPOC) (Jones et al., 2007). A canola oil (CO) diet was used as a negative control because it has no ω3 LC-PUFA (Carter et al., 2003), but has high concentrations of ALA, the precursor to SDA. A fish oil diet (FO) was used as a positive control that represents a traditional aquafeed diet that is rich in EPA and DHA.

6.2 MATERIALS AND METHODS

6.2.1 Experimental Design

Three experimental diets (4 tanks/diet, 25 fish/tank) were fed to Atlantic salmon for 86 days. At the end of the trial, 12 fish from each diet were euthanised and samples of red and white muscle, liver and total carcass were taken for FA analysis. These fish formed part of an experiment investigating the effect of replacing fish oil with

SDA (see Miller et al., 2007). A further 9 fish per diet were taken for metabolic rate analysis, after which samples of heart tissue were collected for FA analysis.

6.2.2 Experimental diets

Three diets were formulated to compare the effects of canola oil (CO), stearidonic acid oil (SO) and fish oil (FO) on the metabolic rate of Atlantic salmon (Table 6.1). Fishmeal was defatted three times using a 2:1 mixture of hexane and ethanol (400ml $100g^{-1}$ fish meal). Stearidonic acid oil was supplied as Crossential SA14 (Croda Chemicals, East Yorkshire, UK). Fish oil was from jack mackerel, *Trachurus symmetricus* L., (Skretting Australia, Cambridge, Tasmania, Australia) and a domestic source of pure canola oil was used (Steric Trading Pty Ltd, Villawood, NSW, Australia). The diets were manufactured into 3 mm diameter pellets using a California Pellet Mill (CL-2), dried and stored at -5°C.

6.2.3 Growth experiment

Atlantic salmon *Salmo salar* parr (≈87.9 g) were obtained from Springfield Fisheries hatchery (Scottsdale, Tasmania, Australia) acclimated for 14 days in 300 L tanks and fed a commercial feed (Skretting). Prior to the experiment the fish were slowly adapted to saltwater over a 21-day period. The tanks were maintained at a constant temperature of 12.0°C under a natural photoperiod. Holding systems consisted of biofiliters, UV sterilizers and a 0.2 μm cartridge filter. Dissolved

oxygen, pH, ammonia, nitrate, nitrite, and salinity were monitored daily to ensure water quality remained consistent throughout the trial. The fish were held in an average of 27.4 ± 0.2 ppt seawater.

At the start of the experiment fish were anaesthetized (50 mg l^{-1}, benzocaine), their weight and fork length measured, and four fish killed to measure initial lipid content and composition. Twenty-five fish were randomly re-allocated into each of twelve 300 L tanks. Fish were individually marked on the ventral surface by a Panjet (Hart et al., 1969). The three diets were fed in quadruplicate by hand at a ration of 1.1% body weight per day (% BW day^{-1}). Every three weeks, for a total of 86 days, all fish were anaesthetized (50 mg L^{-1}, benzocaine) and batch-weighed. Fish were starved on the day prior to weighing. Total feed consumption (kg DM) was estimated from the amount of feed supplied that was not eaten.

At the end of the experiment fish were starved on the day prior to being anaesthetized (50 mg l^{-1}, benzocaine) and their weight and fork length measured. The first three fish sampled from each tank that had doubled their initial weight were killed by a blow to the head after immersion in anaesthetic. Only fish that doubled their initial weight were sampled. Samples of liver, red and white muscle tissue were dissected from below the dorsal fin and frozen at -80°C until analysis. A further two fish, which had doubled their initial weight, per tank were taken and killed (as described above) and frozen for total carcass analysis.

Specific growth rate (SGR) was calculated as SGR (% day^{-1}) = 100 x (ln (W_f/W_i)) x d^{-1} where W_f and W_i are the final and initial weights (g) and d is the

number of days of the experiment. Total feed consumption (FC) was calculated as the total amount (g DM) consumed per tank over 84 days.

6.2.4 Chemical analysis

All lipid extraction, isolation techniques and crude composition analysis have been described by detail in Miller et al. (2007). Basically, lipid was extracted by freeze-drying the samples, then extracted from the samples using a modified Bligh and Dyer protocol (Bligh and Dyer, 1959). Fatty acid analysis was then determined by gas chromatography mass spectrometry.

6.2.5 Metabolic rate measurements

Routine and maximum metabolic rate measurements were taken following 86 days of pre-feeding of the diets. Metabolic rate measurements were conducted in black acrylic boxes with a mean (± SEM) volume of 2153 ± 13 mL (Jones et al., 2007). Food was withheld for 24 h prior to metabolic rate measurements to ensure that fish were in a post-absorptive state (Jones et al., 2007). Nine fish from each diet were randomly selected for metabolic rate measurements. Fish were placed into respirometry boxes 16 h prior to measurement. Water was supplied to individual respirometry boxes via a 400 L recirculation system channelled through a manifold

Table 6.1.

Ingredient and lipid composition (g/kg dry matter) of experimental diets (CO – canola oil diet, SO – stearidonic acid oil diet, FO – fish oil diet).

	Diet		
	CO	SO	FO
Ingredient composition (g kg^{-1})			
Fish meal (defatted)[1]	150	150	150
Casein[2]	150	150	150
Wheat gluten[3]	100	100	100
Soybean meal[4]	180	180	180
Fish oil[1]	200	0	0
Canola oil[5]	0	200	0
SDA oil[6]	0	0	200
Pre gel starch[7]	127	127	127
Vitamin mix[8]	3	3	3
Mineral mix[9]	5	5	5
Stay C[10]	3	3	3
Chlorine chloride[11]	2	2	2
Sipernat[12]	40	40	40
CMC[11]	10	10	10
Sodium mono phosphate[11]	20	20	20
Yttrium oxide[11]	10	10	10
Chemical composition (g kg^{-1} DM)			
Dry Matter	940.1	943.1	938.1
Crude protein	351.7	340.0	344.1
Crude fat	247.9	255.2	250.8
Energy (MJ kg^{-1} DM)	19.3	19.0	19.1
FAME (g kg^{-1} DM)			
Total SFA	22.1	25.7	68.2
Total MUFA	114.6	41.2	53.7
18:3ω3 ALA	13.1	52.3	1.2
18:4ω3 SDA	0.3	20.0	1.8
20:5ω3 EPA	0.0	0.0	24.2
22:5ω3 DPA	0.1	0.0	2.5
22:6ω3 DHA	0.0	0.2	18.2
Total ω3	13.5	72.5	49.9
18:2ω6 LA	45.2	42.1	11.6
18:3ω6 GLA	0.3	17.1	5.3

Total ω6	49.0	60.3	20.3
Total PUFA	63.2	132.8	77.1

[1] Skretting Australia, Cambridge, Tasmania, Australia

[2] MP Biomedcals Australasia Pty Ltd, Seven Hills, NSW, Australia

[3] Starch Australasia, Lane Cove, NSW, Australia

[4] Hamlet Protein A/S, Horsens, Denmark

[5] Croda Chemicals, East Yorkshire, UK

[6] Steric Trading Pty Ltd, Villawood, NSW, Australia

[7] Penford Australia Limited, Lane Cove, NSW, Australia

[8] Vitamin mix (ASV4) supplied per kilogram of feed: 2.81 mg thiamine HCL, 1.0 mg riboflavin, 9.15 mg pyridoxine HCL, 25 mg nicotinic acid, 54.35 mg calcium D-pantothenate, 750 mg myo-inositol, 0.38 mg D-biotin, 2.5 mg folic acid, 0.03 mg cyanocobalamin, 6350 IU retinol acetate, 2800 IU cholecalciferol, 100 IU DL α-tocopherol acetate, 5 mg menadione sodium bisulphate by Sigma-Aldrich, Castle Hill, NSW, Australia and 100 mg Roche rovimix E50.

[9] Mineral mix (TMV4) to supplied per kilogram of feed:117 mg $CuSO_4.5H_2O$, 7.19 mg KI, 1815 mg $FeSO_4.7H_2O$, 307 mg $MnSO_4.H_2O$, 659 mg $ZnSO_4.7H_2O$, 3.29 mg Na_2SeO_3, 47.7 mg $CoSO_4.7H_2O$ by Sigma-Aldrich, Castle Hill, NSW, Australia

[10] L-Ascorbyl-2-polyphosphate Roche Vitamins Australia, French Forest, NSW, Australia.

[11] Sigma-Aldrich, Castle Hill, NSW, Australia

[12] Degussa, Frankfurt, Germany

SO, stearidonic oil rich diet; CO, canola oil diet; FO, fish oil diet, SFA, Saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; CMC, Carboxymethyl cellulose; DHA, Docosahexaenoic Acid; EPA, Eicosapentaenoic Acid; SDA, Stearidonic acid, ALA, α- Linolenic acid; LA, Linolenic acid: GLA, γ- Linolenic acid.

that allowed separate control of the water flow going into each of the boxes. Water temperature was maintained at 12°C ± 0.5°C with a heat exchange unit, the boxes were also semi-submerged in order to minimize any temperature shift when the flow to the boxes was stopped. Oxygen was maintained at 100% air saturation by an air stone in the header tank.

Prior to all metabolic rate measurements, a thermostatically controlled oxygen electrode (1302 electrode, Strathkelvin Instruments Ltd Glasgow, UK) was calibrated using a 2% $NaSO_3$ (zero) solution and air saturated seawater (155 mm Hg). The electrode was attached to a Strathkelvin Instruments model 782 O_2 meter. To determine routine metabolic rate ($Mo_{2\ rout}$), water and airflow to the boxes was stopped and the box sealed. A 3 mL sample of water was removed from the box and injected into the oxygen electrode. After 10 min a final water sample was taken and the oxygen content re-measured using the same electrode (Powell et al., 2005).

Maximum metabolic rates ($Mo_{2\ max}$) were measured using protocols similar to that found in Cutts et al. (2002). Briefly, individual fish were individually placed into a 50 L cylindrical container that contained hyperoxic seawater (120% saturation, 15°C), measured using a Handy Gamma Oxy Guard, (Birkrød, Denmark), the salmon were chased by hand to exhaustion and immediately returned to the respirometer boxes and their oxygen consumption rate measured (as above) (Cutts et al., 2002). Subsequent MO_2 measurements were taken at 15-minute intervals for the first hour then every hour for 6 hours after chasing to determine EPOC. Metabolic rate was calculated using the following formula:

$$MO_2 = \frac{((PO_{2i} - PO_{2e}) * \alpha) * (V - M)}{T * M} \qquad [1]$$

where PO_{2i} and PO_{2e} are the initial and final oxygen tensions respectively (mm Hg), α is the molar O_2 solubility in water ($\mu M\ O_2\ L^{-1}$ mm Hg^{-1}), V is the respirometer box volume (L), T is the time between the initial and the final oxygen measurements (s) and M is the mass (g) of the fish (Cameron, 1986; Cech, 1990). There was not a complete seal between the air-water interface, the oxygen transfer rates were found to be 0.183 mmHg over a 10 min period, the results were corrected accordingly. Total EPOC (mg O_2 kg^{-1}) was calculated by determined by integrating the area bound between the recovery curve and MO_{2rout}. At the end of the experiment fish were euthanised with an overdose of clove oil (0.05%), heart and blood plasma samples were taken for fatty acid analysis.

6.2.6 Statistical analysis

Mean values are reported as plus or minus the standard error of the mean. Normality and homogeneity of variance were confirmed and percentage data were arcsine transformed prior to analysis. Fatty acid concentrations are presented as percentage of a specific FA relative to the total FA concentration, and as a measurement of the total amount of a specific FA found in the organ. For the metabolic rate measurements, a one-way ANOVA was used to detect significant differences between treatments (initial, SO, FO, CO) for routine and maximum metabolic rate, and metabolic scope. When significant differences were found,

Tukey's post-hoc test was used to elicit where significant differences existed. Total EPOC was calculated by integrating the area underneath the graph bounded between $Mo_{2\ rout}$ and the recovery curve, and from the first metabolic rate measurement post chase and the time the recovery curve reached $Mo_{2\ routine}$ (Lee et al, 2003). Total EPOC was compared across treatments using a one-way ANOVA followed by a Tukey's post-hoc test when differences were detected. Furthermore, a one way repeated measures ANOVA was used to determine when metabolic rate for each individual treatment post chase returned to return $Mo_{2\ rout}$ levels. For all FA analysis comparisons between means was by 1-way analysis of variance (ANOVA) followed by multiple comparisons using Tukey-Kramer HSD. Significance was accepted at probabilities of 0.05 or less. Pearson's correlations were performed by first combining all diets and looking at individual FA correlations with $MO_{2\ rout}$, $MO_{2\ max}$ an metabolic scope, correlations were also run on individual FA and MO_2 parameters within each diet treatment. Only statistically significant correlations are reported. Statistical analysis was performed using SPSS for Windows version 11.

6.3 RESULTS

There was no significant difference in the average final weight, fork length or growth between fish fed the three experimental diets (Table 6.2).

Table 6.2.

Growth and efficiencies of Atlantic salmon fed experimental diets with canola oil (CO), 14% stearidonic acid oil (SO) and fish oil (FO) (mean ± SEM). No significant differences were found between any of the treatments using a 1-way ANOVA (n = 9 fish per diet).

	CO	SO	FO
Initial weight (g)	111.28 ± 3.32	112.33 ± 6.17	101.44 ± 4.66
Final weight (g)	157.70 ± 8.10	166.10 ± 5.89	158.31 ± 7.42
Weight gain (g)	46.42 ± 6.6	53.77 ± 7.07	56.87 ± 6.98
Initial length (cm)	21.76 ± 0.1	21.6 ± 0.36	21.66 ± 0.3
Final length (cm)	25.01 ± 0.35	25.18 ± 0.24	25.17 ± 0.34
T. feed cons. (g DM)	2569.0 ± 69.4	2697.6 ± 41.5	2520.9 ± 33.4
[1]SGR (% day^{-1})	0.4 ± 0.1	0.5 ± 0.1	0.5 ± 0.0
[2]Survival	89.0 ± 3.8	76.0 ± 1.4	87.0 ± 5.7

DM, Dry matter

[1]SGR, Specific growth rate = 100 x (ln ($W_{final(g)}/W_{initial(g)}$)) x number of days $(d)^{-1}$

[2]Survival during growth experiment.

Mean values across the rows were not significantly different as determined by Tukey-Kramer HSD.6.3.1 Fatty acid analysis

6.3.1.1 Heart tissue fatty acid composition

Fish oil (FO) fed fish generally had higher concentrations of saturated fatty acids (FA) in the heart tissue than fish fed stearidonic acid oil (SO) and canola oil (CO) diets (Table 6.3). FO fish had significantly higher concentrations of 14:0 and 16:0 than CO and SO fish, whilst SO fish had significantly higher concentrations of 18:0 than CO fish and similar concentrations to FO fish. Fish fed the CO diet had significantly higher concentrations of total monounsaturated fatty acids (MUFA) than FO and SO fish, particularly 18:1ω9. Interestingly, SO and FO fish had similar concentrations of total ω3, both groups had significantly higher concentrations than CO fish. Specifically, SO fish had higher concentrations of 18:3ω3 and 18:4ω3 than CO and FO fish, and FO fish had significantly higher concentrations of 22:6ω3 (DHA) than CO and SO fish. SO fish had significantly higher concentrations of ω6 FA, specifically, SO fish had higher concentrations of 18:3ω6 and 20:3ω6, with CO and SO fish having significantly more 18:2ω6 than FO fish.

Table 6.3.

Fatty acid content and lipid class composition of the heart of Atlantic salmon smolt fed canola oil (CO), stearidonic rich oil (SO) diets and fish oil (FO) (g/100 g total fatty acids) Data is shown as mean ± SEM, $P < 0.05$ indicates significant difference between the treatments using a 1-way ANOVA. (n = 9 fish per diet)

FA $mg.g^{-1}$	CO ± SEM	SO ± SEM	FO ± SEM	sig	f	df
14:0	1.1 ± 0.6	0.5 ± 0.2	1.9 ± 0.5			
16:0	10.2 ± 2.1	10.8 ± 1.8	12.7 ± 1.3			
18:0	4.5 ± 1.3	5.0 ± 0.9	3.8 ± 0.4			
Other SFA	0.9 ± 0.6	0.2 ± 0.1	0.4 ± 0.1			
Total SFA	16.6 ± 4.6	16.4 ± 2.8	18.8 ± 2.2			
16:1w7c	1.7 ± 0.7	0.9 ± 0.2	3.2 ± 0.8			
18:1w9c	25.2 ± 3.9b	11.0 ± 1.8a	8.8 ± 1.7a	0.00	11.0	2,27
18:1w7c	3.7 ± 1.1	1.7 ± 0.3	2.8 ± 0.4			
20:1w9c	2.0 ± 1.3	0.3 ± 0.1	0.3 ± 0.2			
Other MUFA	0.9 ± 0.7	0.0 ± 0.0	0.2 ± 0.1			
Total MUFA	33.4 ± 7.2b	13.9 ± 2.2a	15.3 ± 3.1a	0.01	5.4	2,27
18:3w3	1.8 ± 0.2a	5.1 ± 0.7b	0.4 ± 0.1a	0.00	33.8	2,27
18:4w3	0.7 ± 0.1a	3.3 ± 0.5b	0.8 ± 0.2a	0.00	19.4	2,27
20:4w3	0.0 ± 0.0	0.2 ± 0.1	0.3 ± 0.1			
20:5w3	1.6 ± 0.4a	3.1 ± 0.5a	5.1 ± 0.6b	0.00	12.8	2,27
22:5w3 DPA	0.1 ± 0.1	0.3 ± 0.2	0.8 ± 0.4			
22:6w3 DHA	6.6 ± 0.8a	7.2 ± 1.1a	12.4 ± 1.2b	0.00	9.4	2,27
Other w3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0			
Total w3	10.9 ± 1.3a	19.3 ± 2.7b	19.8 ± 2.2b	0.01	5.4	2,27
18:2w6	8.1 ± 1.0b	7.5 ± 1.0b	2.5 ± 0.5a	0.00	11.8	2,27
18:3w6	0.6 ± 0.1a	1.8 ± 0.3b	0.0 ± 0.0a	0.00	27.2	2,27
20:3w6	0.6 ± 0.2a	1.3 ± 0.2b	0.0 ± 0.0a	0.00	12.7	2,27
20:4w6	1.4 ± 0.2	1.1 ± 0.2	0.9 ± 0.1			
22:5w6 DPA	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0			
Other w6	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0			
Total w6	10.7 ± 1.3a	11.6 ± 1.7a	3.5 ± 0.7b	0.00	12.0	2,27
Other PUFA	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.2			
Total PUFA	21.6 ± 2.4	30.9 ± 4.3	23.6 ± 2.9			
w3/w6	1.1 ± 0.1a	1.7 ± 0.1a	6.1 ± 0.4b	0.00	111.8	2,27

6.3.1.2 FA profiles of tissues taken for Miller et al (2007).

The FA profiles of the liver (not shown), white muscle (Appendix I, Table 1), heart (Table 6.3) gills (not shown) and whole carcass (Appendix I, Table 2) were significantly different (P <0.01) between the three diet treatments. The concentrations of saturated fatty acids (SFA; 14:0, 16:0, 18:0) in the whole carcass of FO fish were significantly higher (P <0.01) than the CO and SO fish. The concentrations of monounsaturated fatty acids (MUFA), particularly 18:1ω9 in all tissues and organs of CO fish was significantly higher (P <0.01) than in SO and FO fish. SO and FO fish had significantly higher (P <0.01) concentrations of total polyunsaturated fatty acids (PUFA) than CO fish in total carcass and white muscle. The ω3/ω6 ratio in FO and initial fish was significantly (P <0.01) higher than the SO and CO fish in all samples.

6.3.2 Metabolic rates

6.3.2.1 Diet Effects

There were No significant differences in routine metabolic rates between any of the treatments (P = 0.367, F = 1.095, df = 3, 30, Table 6.4). Similarly, no significant differences were detected between treatments for maximum metabolic rate (P = 0.151, F = 1.897, df =3, 30, Table 6.4). The metabolic scope of CO fish was significantly lower than that of the metabolic scope of the initial fish, but was not significantly different from the other two treatments (P = 0.052, F = 2.872, df = 3,30, Table 6.4).

There was no significant difference between the treatments for total EPOC ($P = 0.582$, $F = 0.554$, $df = 3, 30$, Figure 6.1). For the initial treatment, Mo_2 returned to $Mo_{2\ rout}$ levels 120 min post-exercise ($P = 0.001$, $F = 25.732$, $df = 3.30$). All other treatments had Mo_2 post-exercise that were not significantly different from $Mo_{2\ rout}$ 45 min post-exercise (FO, $P = 0.001$, $F = 19.112$, $df = 3,30$; SO, $P = 0.001$, $F = 19.123$, $df = 3,30$; CO, $P = 0.001$, $F = 14.917$, $df = 3,30$).

Table 6.4.

Routine and maximum metabolic rate, and metabolic scope of fish fed diets with three different sources of lipids. Initial measurements were taken prior to the feeding of the diets. Superscript indicates significant difference between treatments using a 1-way ANOVA ($P < 0.05$, $n = 9$ fish per diet)

	Treatment			
	Initial	Canola	Fish	SA
$Mo_{2\ rout}$	4.47 ± 0.25	4.67 ± 0.58	4.75 ± 0.58	4.66 ± 0.49
$Mo_{2\ max}$	12.31 ± 0.69	9.89 ± 0.47	11.08 ± 0.40	10.62 ± 0.32
Scope	7.84 ± 0.76[a]	6.69 ± 1.00[b]	5.71 ± 0.59[b]	5.37 ± 0.60[b]

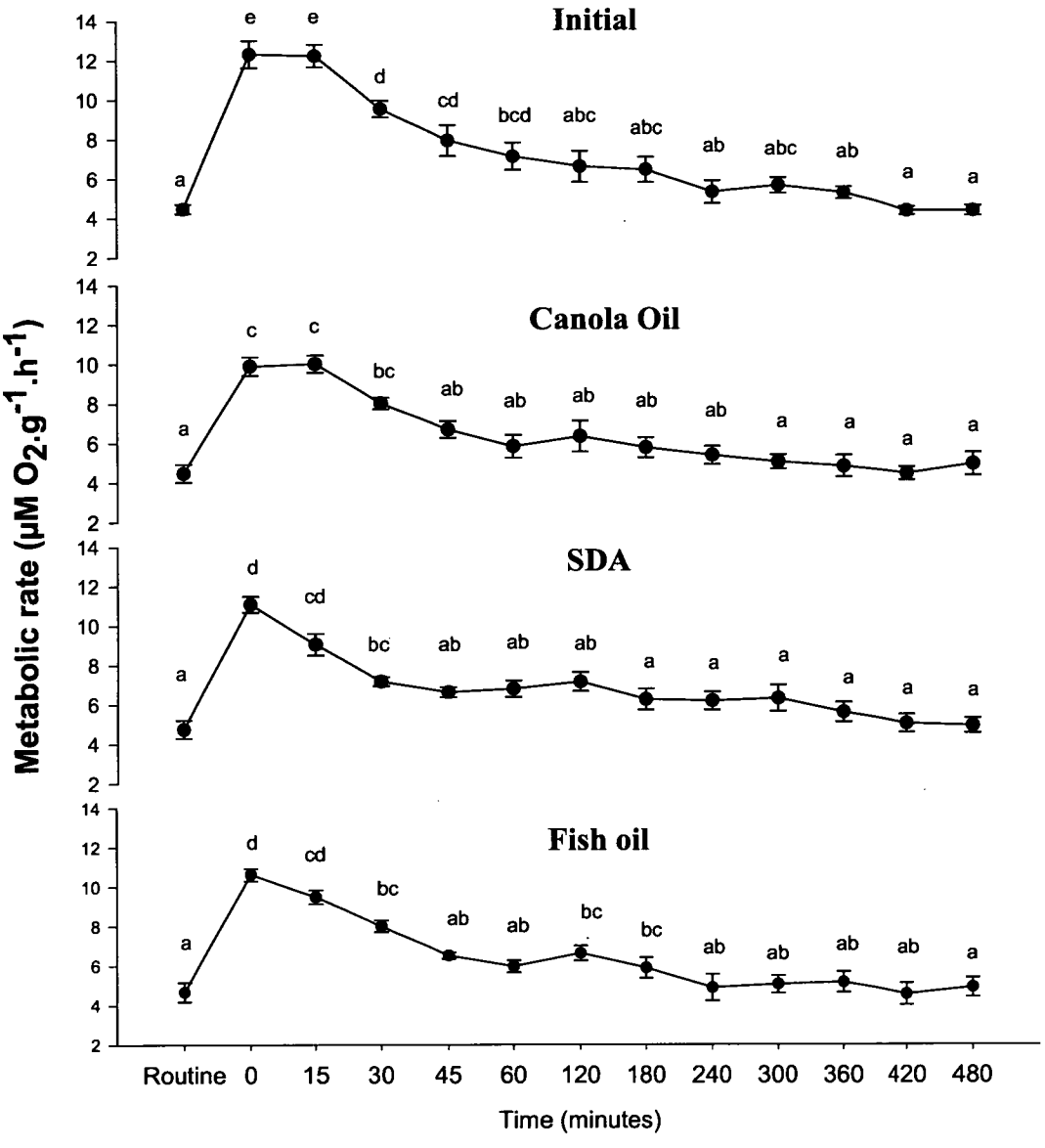

Figure 6.1.

Metabolic rate (μM O_2 .g^{-1}.h^{-1}) of Atlantic salmon smolt fed three different dietary sources of oil (n = 9 fish per diet) pre-exhaustive exercise ($Mo_{2\ rout}$) and 480 minutes post exhaustive exercise. Superscript letters indicate significant difference ($P < 0.05$) from $Mo_{2\ rout}$.

6.3.2.2 Correlations

For individual diets, there were significant negative correlations between $MO_{2\ rout}$ and FA concentrations (Table 6.5) in the heart tissue for fish fed the SO diet. Therewas a significant negative correlation between LA (18:1ω9) in the heart muscle and $MO_{2\ rout}$ for fish fed the CO and SO diets, and a positive correlation between LA and metabolic scope for fish on the SO diets. There was also a negative correlation between EPA, SDA, ALA and total ω3 and $MO_{2\ rout}$ and positive correlations for EPA, SDA, ALA, DHA and total ω3 and ω6 and metabolic scope for fish fed the SO diet. No significant correlations were found for $MO_{2\ max}$ and metabolic scope and any of the FA in the heart muscle tissue, nor was there any correlations found when examining percentage FA and any of the metabolic parameters. For fish fed the FO diet, there was a significant positive correlation between $MO_{2\ rout}$ and 18:3ω3, and a significantly negative relationship between $MO_{2\ max}$ and other ω3 FA (Table 6.5). For fish fed the CO diet there were significant positive relationships between 18:2ω6, 19:3ω6 and total ω6 FA and $MO_{2\ rout}$ /(Table 6.5). There were also negative relationships between 18:4ω3 and 18:3ω6 and metabolic scope.

When data were combined for all diets there were significant positive correlations between $MO_{2\ max}$ and the percentage (g FA/ 100g total FA in tissue) of SFA (16:0, 18:0), EPA, DHA, and negative correlations with the percentage of OA (18:1ω9) and total MFA in the heart (Table 6.6). There were also significant positive

correlations between $MO_{2\ max}$ and ω3/ω6, ω3/OA, DHA/AA and total ω3 content. There were no correlations with percentage FA (g FA/ 100g total FA in tissue) in the tissue and $MO_{2\ rout}$ or metabolic scope.

Table 6.5.

Significant correlation coefficients (P > 0.05) of a number of different fatty acids when examining the total amount of FA (mg total) present in the heart muscle of Atlantic salmon fed the SO (SDA), FO (fish oil) and CO (canola oil) diet as a primary source of FA and $MO_{2\ rout}$, $MO_{2\ max}$ and metabolic scope (n = 9 fish per diet)

FA	Correlation	P	FA	Correlation	P
SDA $Mo_{2\ rout}$			*SDA scope*		
14:0	-0.875	0.002	16:0	0.895	0.001
16:0	-0.832	0.005	18:0	0.867	0.002
18:0	-0.782	0.013	Total SFA	0.892	0.001
Total SFA	-0.843	0.004	16:1ω7	0.673	0.047
16:1ω7	-0.769	0.016	16:1ω9	0.921	0.000
16:1ω9	-0.861	0.003	18:1ω7	0.882	0.002
18:1ω7	-0.783	0.013	Total MFA	0.933	0.000
Total MFA	-0.884	0.002	18:3ω3	0.862	0.003
18:3ω3	-0.817	0.007	18:4ω3	0.879	0.002
18:4ω3	-0.903	0.001	20:5ω3	0.900	0.001
20:5ω3	-0.766	0.016	DHA	0.817	0.007
Total ω3	-0.790	0.010	Total ω3	0.886	0.001
18:2ω6	-0.803	0.009	18:2ω6	0.938	0.000
18:3ω6	-0.870	0.002	18:3ω6	0.879	0.002

20:4ω3	-0.768	0.016	20:4ω3	0.847	0.004
Total ω6	-0.838	0.005	Total ω6	0.919	0.000
Total PUFA	-0.818	0.007	Total PUFA	0.908	0.001
Fish oil $MO_{2\ rout}$			*Fish oil $MO_{2\ max}$*		
18:3ω3	0.858	0.003	Other ω3	-0.679	0.044
Canola Oil $MO_{2\ rout}$			*Canola Oil scope*		
18:2ω6	0.679	0.044	18:4ω3	-0.709	0.033
19:3ω6	0.717	0.030	18:3ω6	-0.707	0.033
Total ω6	0.686	0.041			

Table 6.6.

Significant correlation coefficients (P > 0.05) between $MO_{2\ max}$ and FA content (g/100 g total fatty acids in tissue) and FA ratios in the heart muscle of Atlantic salmon fed 3 different oil based diets (n = 9 fish per diet).

$MO_{2\ max}$

FA	Correlation	P
18:1ω9 (OA)	-0.453	0.018
20:5ω3 (EPA)	0.391	0.044
22:6ω3 (DHA)	0.551	0.003
Total ω3	0.469	0.014
Total MFA	-0.401	0.038
16:0	0.490	0.009
18:0	0.388	0.045
Total SFA	0.437	0.023
ω3/ω6	0.419	0.030
DHA/AA	0.417	0.031
ω3/OA	0.493	0.009

6.4 DISCUSSION

The current study assessed the potential effects on the metabolic rate and recovery post-exercise in Atlantic salmon of replacing the fish oil content of a typical salmon diet with Echium oil that contained a high amount of stearidonic acid (SDA),. Previous studies have shown that replacing fish oils with alternate oil sources can have a profound impact on the metabolic rate and swimming performance of Atlantic salmon, Adriatic sturgeon, seabass and European eels (McKenzie et al., 1998; Wagner et al., 2004, Chatelier et al., 2006). The current study, however, found that replacing fish oil in the diets of Atlantic salmon had little effect on the $MO_{2\ rout}$, $MO_{2\ max}$ and recovery from exhaustive exercise. Similar results were found by Wilson et al (2007) when replacing up to 75% of fish oil with plant-based oils in a diet for Atlantic salmon. Significant correlations were found when analysing the concentrations of specific fatty acids in the heart and $MO_{2\ max}$, suggesting that there may be some relationship between concentrations of specific fatty acids in the heart and exercise physiology in Atlantic salmon.

6.4.1 Whole body and tissue fatty acid analysis

Previous studies have highlighted the effect of differing concentrations of FA, primarily ω3 LC-PUFA and SFA in the diet of teleosts and chondrostean fish can have on standard metabolic rate (SMR). European eels *Anguilla anguilla* fed for 90 days on a diet supplemented with 15% menhaden oil, an oil rich in ω3 LC-PUFA,

had significantly lower SMR's compared to eels that had been fed a diet supplemented with coconut oil, which is rich in SFA (McKenzie et al., 2000). Similarly, Adriatic sturgeon *Acipenser naccarii* fed a commercial diet supplemented with menhaden oil had lower metabolic rates than those of the controls (Randall et al, 1992, McKenzie 2001). A reduction in SMR in European eels and Adriatic sturgeon was associated with significantly increased total ω3 content, ω3/ω6 ratios, EPA/AA ratios and SFA (Randall et al, 1992, McKenzie 2001). In the current study, there were significant differences in total ω3 LC-PUFA content in all of the tissues tested, with fish fed the CO diet having significantly less total ω3 LC-PUFA in the white muscle tissue than the SO diet, although there were no significant differences between CO, initial and FO, nor was there a difference between FO, SO and the initial samples. The FO fish had significantly higher concentrations of EPA and DHA than all other treatments, however, no differences were found between the diet treatments with regards to SFA. However no differences were found in either $MO_{2\ rout}$, $MO_{2\ max}$ nor metabolic scope despite the differences in FA profiles between the diets. The results are similar to Wilson et al (2007) who found no significant differences in $MO_{2\ rout}$, $MO_{\ max}$ and repeat swimming performance when replacing up to 75% of anchovy oil with plant and poultry based oils using similar physiological analysis techniques to those used in the current study.

Why some studies detect differences, whilst seemingly similar studies find none is of interest, and may be due to differences in methods and analyses. Some of the

previous studies where differences were found between diet treatments ran for between 100 days up to a year, considerably longer than the current trial (McKenzie et al., 1997; McKenzie et al., 1998; McKenzie et al., 2000; Chatlier et al., 2006). Furthermore, there where significant differences in weight gain between the current trial and previous trials, for example, Atlantic salmon grew from 140g to 400g (McKenzie et al., 1998), seabass in doubled in weight (Chatelier et al., 2006) whilst Atlantic sturgeon grew from 198g to 1630g over the duration of the study (McKenzie et al., 1997). Atlantic salmon in the current trial grew from approximately 108g to 160g over the course of the trial, a weight gain that is comparatively small when compared that of the aforementioned trials. It is possible that the physiological changes observed in other trials, examining fish oil replacement diets in fish, occur over a time frame and require a weight gain in fish in excess of what occurred in the current study.

Certain combinations of FA may intensify or attenuate the effects of certain individual FA on metabolic rate and swimming performance, thus greatly complicating the assessments of specific dietary treatments on metabolic rate (Wagner et al., 2004). Given that a number of studies have used different control and replacement oils, with quite conflicting results, suggests that predicting a response in metabolic rate and swimming performance based solely on the concentrations of specific FA may not be straight forward, and instead, each FA combination present within any diet be treated on its own merit. It is not known why certain FA's have an influence on metabolic rate and swimming performance.

What is known is that FA have multiple roles in animal cells, phospholipids are a crucial component of cell membranes, triacylglycerols are used primarily as an important energy source and some C_{20} HUFA act as precursors of eiconsanoids that are required for cellular processors (McKenzie 2001). The effect of FA on metabolic rate appear at the fundamental level, with species from widely different taxonomic groups (chondrosts and teleosts) responding to a decrease in ω3 FA in the diet with an increase in SMR. Several theories attempt to explain why a reduction in metabolic rate is associated with increases in dietary ω3-LC PUFA. Changes in ratios of SFA, MUFA and PUFA may cause a reduction or increase in metabolic rate, as fish primarily use FA as an energy source, and preferentially oxidize MUFA>SFA>PUFA (McKenzie, 2001). However in all experiments conducted to date that have examined the effects of FA on metabolic rate, concentrations of MUFA and SFA have been relatively equal between dietary treatments (McKenzie et al., 1997, 2000). A reduction in cardiorespiratory performance has also been noted in treatments that have low concentrations of ω3 LC –PUFA (Bell et al., 1991, 2003, Paige et al., 1996, Randall et al 1992 ,Ushio et al, 1997). Concentrations of ω3 LC-PUFA have also been shown to influence Na^+,K^+-ATPase and Ca^{2+}-ATPase activity, both of which represent a significant proportion of cell energy consumption (Rolfe and Brown, 1997, Gerbi et al., 1994, Ushio et al., 1997, Paige et al., 1996). The effects that specific FA have on the metabolic rate of fish could be a combination of several factors and future research is required to determine the effects that specific FA have on cellular metabolic

processes in order to shed some light on the roles that specific FA have on metabolic rate (McKenzie 2001).

No differences were found in the maximum metabolic rates or metabolic scope of the Atlantic salmon fed different diets. This is in line with the results of Wilson et al (2007) and to some extent the results of Wagner et al (2004). Atlantic salmon fed diets that contain plant-based oils have been shown to have similar initial maximum swimming performance abilities as those fed diets that have predominantly fish oil as a lipid source (Wagner et al., 2004, Wilson et al., 2007). Diets that have high concentrations of ω3 LC-PUFA have been shown to increase tissue oxygen delivery which in turn leads to better cardio-respiratory performance (McKenzie et al.,1995;1999). Maximum metabolic rate is thought to be influenced by the ability of fish to deliver oxygen to the tissues (Jones 1971), therefore an increase in ω3 LC PUFA in the diet would be expected to be reflected in an increase in maximum metabolic rate. Whilst Wagner et al, (2004) found a positive correlation with ω3 LC PUFA in the diet and increased swimming performance, no overall differences were detected in initial swimming performance between fish fed a fish oil based diet and fish fed a flaxseed oil or sunflower oil-based diets, despite fish fed flaxseed or sunflower oil based diets having significantly less ω3 LC PUFA in the white muscle tissue. This suggests that it may not be the amount of ω3 LC PUFA that is important to maximum metabolic rate, rather the combination of specific FA in the diet. Indeed it has been shown in two different species of fish (Atlantic salmon and seabass) that oleic (OA; 18:1ω9) and linoleic acids (LA; 18:2ω6) improve cardiac output, U_{crit} performance and maximum metabolic rate

(Chatlier et al., 2006). In the current study, the CO fish had significantly higher concentrations of OA in the white muscle tissue, whilst the SO fish had significantly higher concentrations of LA than the FO fish. However, this did not translate into an appreciable difference in maximum metabolic rate.

Dietary oils source did not appear to affect EPOC. Furthermore, all fish from all diet treatments had metabolic rates that were not significantly different routine metabolic rates 45 min post-exhaustion. Atlantic salmon primarily use lipids to fuel recovery post exhaustive exercise (Richards et al.,2002), and *in vitro* studies have found that the heart and red muscle tissue preferentially oxidizes MUFA>SFA>ω3 LC PUFA (Henderson and Sargent, 1985; Sidell and Driedzic, 1985; Egginton, 1996). The SO fish had significantly less MUFA than the CO fish but not the FO fish, and there were no significant differences in SFA between the dietary treatments. As there were no significant differences between the treatments with regards to total EPOC, it is possible that concentrations of MUFA within the FO and SO treatments were sufficient, and that MUFA in the CO diet were provided to excess. It is unlikely that the concentrations of MUFA in the FO and SO diets were inadequate, since Atlantic salmon with total MUFA concentrations in white muscle tissue of 21 – 22% total FA content were able to recover fully from exhaustive exercise in order to perform a second swimming performance test within 45 minutes from the previous swimming performance test (Wagner et al 2002). In the current trial the CO, SO and FO diets had percentage total values of MUFA in white muscle tissue of 47.4%, 28.1% and 34% respectively.

6.4.2 Heart muscle correlation data

6.4.2.1 Analysis of FA and ratio's of FA of all diets combined and $MO_{2\ max}$

The effect of reduced dietary concentrations of specific FA has been shown to significantly affect the respiratory and cardiovascular physiology of a number of species of fish (McKenzie, 2001). Atlantic salmon fed diets with a low ω3/ω6 ratio developed severe cardiac lesions and have elevated mortalities following transportation stress (Bell et al., 1991; 2003). Furthermore, *in-vitro* work on the hearts of Adriatic sturgeon that were fed a diet supplemented with fish oil found that the maximum scope for work, measured as cardiac output (Q), was almost twice as much as the hearts of fish fed a diet rich in coconut oil which is high in SFA (Agnisola et al., 1996). Cardiac performance is intrinsically linked with maximum swimming performance (U_{crit}) and $MO_{2\ max}$, as cardiac output increases so does the capacity for aerobic swimming, up until a point at which oxygen demand from the skeletal muscle tissue exceeds the oxygen supply via circulation (Farrell, 2002). Recent work with rainbow trout has shown that intrinsic individual variation in Q_{max} can be linked to similar diversities in $MO_{2\ max}$, metabolic scope and U_{crit} (Claireaux et al., 2006). Furthermore, both cardiac output and MO_2 reach a plateau as fish approach maximum U_{crit}, giving evidence to suggest that it is cardiac output that is limiting aerobic scope (Farrell, 2002, Chatelier et al., 2006). Therefore any change in cardiac output through inadequate dietary supply of ω3 LC-PUFA, could possibly affect swimming performance and $MO_{2\ max}$. The results of the

current trial mirror the results found by Wagner et al. (2004), who found a positive relationship between U_{crit} and EPA, DHA, ω3/ω6, ω3/OA, and DHA/AA, and a negative relationship with OA and U_{crit}, and no relationships with U_{crit} and LA. However unlike Wagner et al. (2004), no relationships were found between swimming performance and ω3/SFA and EPA/AA. The present results support the findings of McKenzie et al (1993;1999) and Wagner et al (2004) that high concentrations of ω3 LC-PUFA are beneficial to the maximum swimming performance of fish.

6.4.2.2 Analysis of total FA in heart muscle of fish fed the SO diet.

The results from the correlation data for $MO_{2\ rout}$, metabolic scope and FA concentration in the heart muscle of fish fed the SO diet presents some interesting possibilities. Fish fed the SO diet were fed a diet deficient in EPA and DHA, and as such any EPA and DHA in heart during $MO_{2\ rout}$ measurements must have either been present prior to the experiment and retained, or endogenously synthesized from dietary precursor fatty acids, in particular stearidonic acid (SDA). Fish with the lowest $MO_{2\ rout}$ had the highest retention of EPA and DHA in the heart. Prior to being fed the SO diet all fish were fed a diet that contained fish oil, therefore all fish prior to been fed the experimental diets had a certain amount of EPA and DHA in their tissues. Miller et al (2007) showed that there was an increase in gene expression in the liver for the genes involved in the ω3 LC-PUFA biosynthetic pathway for fish fed the SO diet, this was reflected in fatty acid profile

with increase EPA concentrations in the liver, however concentrations where not comparable to those fish on the FO diet. However when looking at the concentrations of EPA and DHA in the various organs, it becomes apparent that the concentrations of EPA and DHA in fish fed the SO diet are not evenly distributed amongst the organs, possibly due to the functionality of these organs. White muscle tissues had the least amount of EPA and DHA, followed by the gill, liver then the heart with the red muscle tissue having the highest concentration of EPA and DHA. This suggests that fish may preferentially retain certain FA in certain organs in order to maximize the performance of that particular organ. Functional tissues such as the heart have a requirement for certain concentrations of ω3 LC-PUFA, previous studies have shown that salmon fed diets deficient in ω3 LC-PUFA presented with cardiac lesions and have a reduction in cardiac performance, as well as a reduction in physiological performance associated with swimming (Agnisola et al., 1996; Wagner et al., 2004). Salmon fed the SO diet may have preferentially retained/produced EPA in the heart muscle tissue by bypassing the rate limiting Δ^6 desaturase step, thus increasing biosynthesis in the tissues (Miller et al., 2007). This increase in EPA and to some extent DHA may have lead to an increase in cardiac performance and thus a decrease in $MO_{2\,rout}$ and an increase in metabolic scope.

6.4.2.3 Summary

In the present study no differences in any of the metabolic parameters measured were detected between any of the diet treatments when looking at the dietary

treatments and MO_2 at a group level. This is in contrast to previous studies that have shown that the fatty acid profile of the diet can have profound effects on the metabolic rate of fish, however the results agree with the conclusions of Wilson et al (2007) who found fish oil replacement had no effect on physiological performance. Significant correlations where found between certain individual FA concentrations in the heart and MO_2 measurements, suggesting that analysis of MO_2 on the individual level is a much more robust method of analysis. There are certain limitations that one can place on the conclusions drawn from this study due to limitation in experimental design. It is suggested that for future trials examining similar variables that measurements of $MO_{2\ rout}$ be made over a considerable period of time, more than 8 hours, in order to be confident in the results obtained. The research also highlights the need for measuring individual metabolic rates and correlating those measurements with the FA profile of that particular fish.

Chapter 7 - General Discussion

7.1 Preamble

The principle aim of this research was to examine the metabolic cost of gill diseases, and the possible interaction between nutritional status, disease and metabolic rate and swimming performance. The research also aimed to further knowledge on the physiological mechanisms underpinning the changes in metabolic rate associated with disease. The effect of a fish oil replacement diet on the metabolic rate and metabolic scope for Atlantic salmon was also described. The work contained within this thesis documented changes in metabolic rate associated with two gill diseases that have distinctly different modes of action (Chapters 2 and 3), and has provided the first direct evidence of a change in protein synthesis rates associated with a gill disease (Chapter 5). This Chapter will discuss the major findings and implications of this research and will highlight potential avenues for further study.

7.2 Determining the energetic cost of disease (Chapter's 2, 3 and 5)

One of the major aims of this thesis was to quantify the impact of two distinctly different gill diseases on the metabolic rate of Atlantic salmon, this was achieved by measuring the increase in $MO_{2\,rout}$ associated with disease. In order to calculate the energetic cost of disease in kilojoules, an oxycalorific coefficient of 13.56 J. mg O_2 consumed $^{-1}$ was assumed (Brafield, 1985). To determine the overall energetic cost

of disease for a growing fish, the following conditions were assumed; that a 100 g fish was eating at 1% body weight per day of a 21.4 kJ/g feed, of which 85% of the energy from the feed was digestible, under these conditions the fish was consuming 18.2 kJ day^{-1} of energy (Carter, 2007). All The following discussion will be based on the fed infected group, because this is the most relevant group to aquaculture conditions as fish in aquaculture situations rarely encounter periods of famine.

7.2.1 *Tenacibaculum maritimum*

For the 100 g fish infected by *Tenacibaculum maritimum* (Chapter 2), the average routine energy consumption of a quiescent feeding fish in a post absorptive state prior to infection was 6.4 kJ day^{-1}, this increased to 8.6 kJ day^{-1} post infection. Based on the aforementioned assumptions, routine energy consumption rates prior to infection represented 35.2 % of the total energy consumed, post infection this increased to 47.3 % of the total energy consumed, a 12.1 % increase.

7.2.2 AGD

For the AGD trial, the average routine energy consumption of fish prior to infection was 4.9 kJ day^{-1} for fed infected fish, 20 days post infection this figure rose to 8.6 kJ day^{-1}, an average increase of 3.7 kJ day^{-1}. Routine energy demands prior to infection represented 27.1 % of the total energy consumed during the day, whilst on day 20 of the infection energy demands rose to 47.1 % of the total energy consumed, an increase of 20.1 %. This suggests that up to 20.1 % of the total

energy being consumed goes to servicing the metabolic costs of AGD. The results from Chapter 5 showed that there was an increase in protein synthesis in the gills of affected Atlantic salmon associated with AGD. The average energy consumption of the control fish associated with gill protein synthesis was 0.08 kJ day^{-1} or 0.5% of the total daily energy intake, for infected fish this rose to 0.12 kJ day^{-1} or 0.6% of the total daily energy intake, an increase of just 0.1%. The increase in protein synthesis in the gills of affected salmon only occurred at the organ level, there was no change in white muscle protein synthesis rates suggesting that protein synthesis rates of the entire animal remained stable. The small increase in total daily energy intake associated with AGD attributed protein synthesis is predominantly due to the small percentage of the total body mass that the gill occupies. Generally, gill tissue represents 4-5% of the total mass of the fish, so even large increases in protein synthesis rates in the gills are unlikely to affect the energy consumption rate of the entire organism (Lyndon and Houlihan, 1998). It is highly likely that the increase in energy consumption associated with AGD found in chapter 3 is more likely to be associated with the cardiovascular changes observed in previous studies (See Chapter 3, discussed below). That the increase in metabolic rate associated with gill diseases is so high, yet the increase in gill oxygen consumption, and hence energy consumption is so small, indicates the importance of gill tissue in maintaining a number of physiological functions that can affect the entire organism. Fish gills are the major organ for respiratory exchange, are important for maintaining osmotic and ionic balances and are the main site for nitrogenous excretion (Mommsen, 1984; Jobling, 1994). Whilst gill tissue doesn't necessarily consume a large

proportion of the total oxygen consumed by the fish, the results suggest that any change in the physiological structure or capabilities of the gill will ultimately have wide reaching physiological implications for the entire organism.

Although both AGD and infection with *T. maritimum* both elicited an increase in $MO_{2\ rout}$, the mechanisms behind the increase are likely to be different. Fish infected by *T. maritimum* showed extensive epithelial necrosis of the gill tissue that was directly exposed to *T. maritimum*. Further to this, there was a significant increase in plasma osmolality, suggesting that the necrosis induced by *T. maritimum* resulted in an osmotic failure (Powell et al., 2005). Given the large role that gill tissue plays in osmoregulation, and the significant metabolic cost that is associated with maintaining osmotic balances, it is of little surprise that a necrotic gill infection resulting in osmotic failure lead to an increase in metabolic rate. Fish affected by AGD also showed a significant increase in $MO_{2\ rout}$. The fish in this study displayed the typical multifocal hyperplasic lesions caused by the fusion of lamellae and hyperplasia of the filamental epithelium. It was thought that the increase in metabolic rate observed in Chapter 3 could be explained by the increase in cell proliferation, quantified as an increase protein synthesis observed in Chapter 5, cellular proliferation being relatively energetically expensive. However, despite a significant increase in gill protein synthesis rates observed in Chapter 5, the extent of the increase in protein synthesis rates in gill tissue only represents a small proportion of the increase in energetic demands associated with AGD observed in Chapter 3. Instead, it is likely that a combination of effects, particularly the

cardiovascular effects reported in Leef et al (2005a; 2005b) and Powell et al (2002) that may be contributing to the increase in $MO_{2\ rout}$ observed in the current trials. The results of Chapter 2 and Chapter 3 suggests that the effect of gill diseases on $MO_{2\ rout}$, at least in Atlantic salmon, is similar for both the proliferative and necrotic diseases studied, although the underlying physiological mechanisms that are driving the change in metabolic rate may be inherently different.

Another possibility is that the increase in $MO_{2\ rout}$ represents the energetic cost associated with mounting an immune response to the disease. An immune response has been detected in fish infected with *T. maritimum* and affected by AGD, and forms the basis of research into vaccine development for both diseases (Gross et al., 2004; Romalde et al., 2005; Toranzo et al., 2005; Bridle et al., 2006). Mounting an immune response is thought to be an energetically demanding process, which necessitates trade off between demands from other energetically expensive processes such as growth and reproduction (Sheldon and Verhulst, 1996). The general response to infection is for an infected organism to shift into a phase of hypermetabolism and protein malnutrition, and to redistribute energy and resources away from anabolic and maintenance processes to metabolic processes driving immunity (Lochmiller and Deerenberg, 2000). The primary response to infection in vertebrates is to activate the innate arm of the immune response, which includes activation of macrophages (Lochmiller and Deerenberg, 2000). Macrophages have been shown to be highly metabolically active, showing similar oxygen consumption rates to heart muscle cells operating at maximum capacity (Newsholme and

Newsholme, 1989). The increase in energy requirements is fuelled by an acceleration in lipolysis, proteolysis and glycolysis, thus prolonged infection can lead to a substantial loss in body weight (Lochmiller and Deerenberg, 2000). This is further exacerbated by the tendency of infected organisms to shift into a phase of anorexia (Baracos et al., 1987). From a whole organism perspective, the increase in energetic demands from mounting an immune response to an infection can be severe. In humans for example, severe infections can lead to a 25-50% increase in routine metabolic rates (Long, 1977; Kreymann et al., 1993). Similar results have been found for laboratory rats (28% increase; Cooper et al., 1994) laboratory mice (30% increase; Demas et al., 1997) and sheep (10 – 49 % increase; Baracos et al., 1987). Interestingly, up to 50% of the increase in metabolic rate in humans is attributed to an increase in protein synthesis, primarily due to the increase in inflammatory proteins and antibodies (Borel et al., 1998). There is little research into the effect of infection on metabolic rate in ectothermic organisms, however there is some direct and indirect evidence that suggests that the cost is on a similar magnitude to that observed in endotherms. Of the studies that have specifically examined infection and metabolic rate, Muchlinski (1985) found a 62.5% increase in $MO_{2\ rout}$ in goldfish *Carrasius auratus* and desert iguana *Dipsosaurus dorsalis* injected with *Aeromonas hydrophilia*, and a 70% increase for green tree frogs *Hyla cinerea* injected with the same bacteria. Some studies have used indirect evidence to come to similar conclusions, female Mallee dragons *Ctenophorus fordi* injected with and *E.coli* lipopolysaccharide (LPS) prior to mating had a reduced reproductive investment in the form of a reduced egg mass size, suggesting that

energy investment that would otherwise have gone to developing larger eggs instead went to the immune response to the LPS (Uller et al., 2006).

Life history theory states that because resources are finite, at least for animals in the wild, investment into one trait, in this case a physiological response to disease, will lead to less resources for other traits with the optimal balance being the one that produces the highest lifetime fitness (Roff, 1992; Stearns, 1992). It is expected that the energetic cost of maintaining immune function and physiological homeostasis would come at the cost of other functions, particularly growth, given the particular importance of maintaining immune function and physiological homeostasis in response to disease to ensure the ongoing fitness of the animal (Uller et al., 2006). The cost of disease on growth will inevitably be more than that directly incurred from the energetic cost of the hosts response, primarily because animals that are affected by disease usually undergo a period of anorexia (Baracos et al., 1987). A reduction in the voluntary food intake is a major factor contributing to the negative energy balance often observed in animals suffering from illness (Baracos et al., 1987). Under normal conditions, a reduction in feed consumption would lead to a reduction metabolic rate, however, as demonstrated in Chapter's 2 and 3, as well as in numerous other studies (see reviews Baracos et al., 1987; Kluger et al., 1998), disease is often accompanied with an increase in metabolic rate. The increase in metabolic rate coupled with a decrease in energy consumption will inevitably lead to a period of negative energy balance, that will result in the cumulative loss of body energy stores and represents a significant cost (Beisel, 1977; Long 1977;

Wilmore and Kinney, 1981). The net result being a decrease in growth efficiency, and thus decreased production and profitability of any aquaculture business.

An often observed phenomena in ectothermic animals affected by a pathogen is a behavioural fever (Kluger et al., 1998). Whilst ectothermic animals such as fish cannot use intrinsic effectors to establish fever, they can produce and maintain fever through behavioural adaptations, primarily by moving to a warmer microclimate (Reynolds et al., 1978; Kluger et al., 1998; Sousa do Amaral et al., 2002). This has been demonstrated in a number of species resulting in a subsequent increase in survival, for example the desert iguana *Dipsosaurus dorsalis* when injected with live *Aeromonas hydrophila* had significantly higher survival rates when incubated at febrile temperatures than those incubated at afebrile temperatures (Kluger et al., 1975), similar results have been found for box turtles *Terrapene carolina* (Sousa do Amaral et al., 2002), locusts (Elliot et al., 2005) and crayfish (Casterlin and Reynolds, 1980). Behavioural fever has also been demonstrated in fish, goldfish *C. auratus* injected with live *A. hydrophilia* and placed in a shuttlebox containing a temperature gradient actively selected temperatures that allowed them to maintain a fever 5°C above ambient, resulting in a significant reduction in mortality (Covert and Reynolds, 1977). An increase in body temperature will inevitably lead to an increase in metabolic rate as metabolic rate and temperature are intrinsically linked (Jobling, 1994). Toads *Bufo marinus* injected with LPS showed a significant increase in metabolic rate, which was associated with behavioural fever (Sherman and Stephens, 1998). The observed increase in body temperature, and hence

metabolic rate, in the aforementioned studies on ectotherms only occurred because the animals were permitted to select their preferred body temperature. Therefore it is possible that the metabolic cost of disease in fish in the wild could be significantly higher than that observed in laboratory trials, as the metabolic cost of disease in laboratory studies, including studies contained within this thesis, are usually conducted in a stable environment, one in which temperature is maintained at a constant. However diseased fish in the wild may select warmer waters, thus inducing fever and raising metabolic rates above those that would be detected in laboratory trials conducted at set temperatures. This would effectively lead to an under-estimation of the metabolic cost of disease in fish (Muchlinski, 1985). This is something that should be taken into account for future research studies attempting to determine the metabolic cost of disease in fish, particularly when examining the effect of disease on wild populations.

A direct application of the observed results in chapter's 2 and 3 to field based situations is not recommended for a number of reasons, primarily because the severity of infection in experimental trials is significantly higher than would be observed in commercial aquaculture. Infection with *Tenacibaculum maritimum* progressed to mortality in a number of fish in the trial in under 48 h, whilst 20 days post infection some fish in the amoebic gill disease trial had up to 80% of all gill filaments with AGD lesions. Amoebic gill disease in Atlantic salmon around the Huon region of Tasmania generally progresses over a number of months and varies in severity depending on water temperature and salinity, and is viewed as a chronic

rather than an acute infection (Adams and Nowak, 2003). Fish that are regarded as having a high level of infection on farm sites typically have less than 20% of all gill filaments with AGD lesions (Adams and Nowak, 2003). It is generally regarded that laboratory induced infections are more aggressive than those found in the field, primarily because of higher inoculation rates (Munday et al., 2001, Gross et al., 2004). The fishes response to a chronic infection, in which they are exposed to a small but constant wild population of the infective agent may be inherently different to that of an acute infection, in which they are exposed to a single high dose of the infective agent. Indeed it was speculated that the lack of an observable immune response in Atlantic salmon exposed for a second time to *Neoparamoeba* spp following a freshwater bath may have been due to the aggressive nature of the infection model (Gross et al., 2004). The extent of the effect of a gill disease on the physiology of a fish may also be subject to a weight/size scaling effect. As fish increase in size the surface area of the gill decreases in proportion to body weight, the effect of gill diseases may therefore be more pronounced in larger production fish than in the smaller experimental fish used in the current trials, as the gill area affected by disease represents a larger loss of surface area relative to body size in larger fish (Rao, 1968; Lyndon and Houlihan, 1998). Therefore it is difficult to translate the physiological response of Atlantic salmon in a laboratory situation to that which occurs in a field type situation. However this particular study provides the foundations for further research examining the metabolic cost of gill diseases, particularly AGD, in farmed salmon. The results of chapter 3 demonstrated that AGD has an effect on $\dot{M}O_{2\ rout}$, further research in farm type situations will give

greater clarity as to the extent of the effect of AGD on $MO_{2\ rout}$ in a farm type situation, in that sense this research acts as a guide for what is to be expected in a farm based situation. The research also outlines the specific methods that should be used in order to maximise the chances of detecting any changes in metabolic rate associated with AGD should further research be conducted.

No significant differences in $MO_{2\ rout}$ were found between control fish and AGD affected fish in Chapter's 4 and 5, despite differences being detected in $MO_{2\ rout}$ in Chapter 3. This may have been due to significant differences in the methods used to detect changes in $MO_{2\ rout}$ for fish affected by AGD between the chapters. The $MO_{2\ rout}$ of fish in Chapter 3 were tracked over the course of the infection by individually tattooing the fish and measuring $MO_{2\ rout}$ prior to infection and at two sample points post-infection, results were expressed as a change in $MO_{2\ rout}$ from the initial measurement. In Chapters 4, 5 however, $MO_{2\ rout}$ was measured only at the end of the experiment, and differences in $MO_{2\ rout}$ between control fish and AGD affected fish were analysed based on the $MO_{2\ rout}$ of the group of fish, rather than changes in $MO_{2\ rout}$ of individual fish. Measuring the effect of a treatment by first determining the response of the organism to a control environment, then exposing it the to the treatment has been used to great effect in a number of studies, for example studying thermoregulation in response to fever in box turtles and crayfish (Casterlin and Reynolds, 1980; Sousa do Amaral et al., 2002). However this method has rarely been adopted in studies examining the effect of disease on the metabolic rate of fish. It would not be possible to measure the metabolic rate of fish prior to infection

in some experiments due to inherent restrictions in experimental design, particularly when studying the effects of disease in wild fish (Farrell and Tierney, 2004; Wagner et al., 2005). Furthermore, measuring the same fish throughout an infection precludes the ability to lethally sample the fish until the final metabolic rate measurement occurs, thus requiring the use of satellite fish within the same tank in order to make assessments on the severity of infection. Therefore caution must be adhered to when examining the effects of disease on metabolic rate in cases where there are wildly different values of disease severity within a given population of fish, as fish sampled to gather information on disease progression may not represent the level of disease present in the fish being used for metabolic rate measurements. Nevertheless, it is recommended that in experiments attempting to elucidate the effect of disease on metabolic rate in fish, that metabolic rate be measured prior-to and post-infection in trials in which infection levels are relatively uniform across a given population, as evidence presented in this thesis suggests that this is a much more sensitive method to determine the effects of disease on metabolic rate.

7.3 Maximum metabolic rate and swimming performance

Neither AGD nor *Tenacibaculum maritimum* had a significant affect on maximum metabolic rate. Both diseases effectively reduce the available surface area for respiration, either through necrosis or by increasing the water/blood interface distance; previous work by Duthie and Hughes (1987) has shown that a reduction in gill surface area can reduce the maximum metabolic rate of fish. However, the

methods used for determining maximum metabolic rate throughout this thesis may not be the most appropriate for elucidating the effect of gill diseases on maximum metabolic rate, primarily because the methods used induce a burst swimming type activity or an unsteady swimming action (Jobling, 1994; Reidy et al., 1995; Burgetz et al., 1998). Burst type swimming requires the fish to be in a constant state of acceleration, fish swimming in such a manner will encounter much higher resistance from the surrounding environment thus greatly increasing the power requirements of the fish per unit time, this will ultimately lead to a greater recruitment of anaerobic metabolism in a much shorter time frame than that of a fish swimming in a steady manner (Reidy et al., 1995). The energy provided for burst type swimming comes predominantly from anaerobic metabolism, the energy requirements of fish swimming in such a manner will be over and above that which can be provided by aerobic metabolism (Burgetz et al., 1998). During anaerobic metabolism fish will predominantly used high-energy phosphate compounds (creatine phosphate) which are quickly exhausted, energy will then by derived from the anaerobic metabolism of muscle glycogen stores, the end product of which is lactate, the accumulation of lactate in the muscle tissue will ultimately lead to the cessation of swimming (Jobling, 1994). Any restriction on the gill surface area of a fish swimming in an unsteady manner will only affect the proportion of energy gained from aerobic metabolism, which as previously stated, during burst type activity is relatively small. The non-aerobic capacity of the fish is better assessed by measuring the fishes ability to recover post-exhaustive exercise, or excess post exercise oxygen consumption (EPOC; see Chapter 4), which is the oxygen required

post exercise to convert lactate back to glycogen, restoring ionic and acid-base balances and recover ATP and oxygen stores (Reidy et al., 1995). Fish swimming in an unsteady manner will predominantly use anaerobic metabolism as a primary mechanism for gaining energy, this may be more so the case in fish that have a reduction in gill surface area. This may lead to greater EPOC, as a greater proportion of anaerobic metabolism is required to meet the energy demands of the fish whilst swimming, this is thought to be the reasoning behind the increase in EPOC of fish affected by AGD observed in Chapter 4. Further adding to this, the increase in the blood-water diffusion distance and the reduction in cardiac output that is associated with AGD lesions (Powell et al 2000; Leef et al., 2005), may effectively limit or prolong the ability of fish to recover to routine levels post-exercise, as the ability to recover is limited by the blood-water diffusion distance, the oxygen carrying capacity of the blood, the surface area of the gill, cardiac output and the diffusivity constant of the gas (Powell, 2007). It is therefore recommended that in addition to determining the effect of disease on the maximum metabolic rate of fish, that a measurement of EPOC be incorporated into the testing methods. Furthermore, it is the author's opinion that swimming performance tests (Chapter 4) give a greater insight into the patho-physiological effects of gill diseases in actively swimming fish primarily because they test the maximum aerobic and anaerobic capacity of the fish, and thus the factor (aerobic capacity) that is most likely to be affected by a reduction in gill surface area (Reidy et al., 1995). Swimming performance tests also allow the individual performance of the fish to be quantified during the exercise protocol, this cannot be done in chase-based

protocols used in the current trials (Reidy et al., 1995). In addition, the results obtained in Chapter 4 highlight the possibility of incorporating two recovery ratio tests on the same fish separated by a short interval of time. This is of particular interest to studies that are examining the effect of a specific disease treatment, in this case a freshwater bath, on the physiology of fish, and may present an alternative method of assessing the effectiveness of a treatment for disease.

7.4 Effect of dietary oil source on metabolic rate and recovery in Atlantic salmon

Chapter 6 highlighted the interaction between the fatty acid composition of the diet, metabolic rate and recovery. There were significant differences in the FA composition of the white muscle tissue and the carcass between the diets, however no differences were found for $MO_{2\ rout}$. Furthermore, there was no difference in growth performance between the diets and major determinants of respiration such as feed intake and growth (protein synthesis) were therefore the same across the diets. In the literature there is a disparity between studies examining the effect of oil replacement on metabolic rate. European eels *Anguilla anguilla* and Adriatic sturgeon *Acipenser naccarii* fed diets rich in ω3 PUFA's had significantly lower $MO_{2\ rout}$ than fish fed diets supplemented with coconut oil (McKenzie et al., 1995; 1997; 2000). Alternatively, studies by Wilson et al (2007) and Chatelier et al (2006) found no effect on $MO_{2\ rout}$ when fish oil was replaced with poultry fat, flaxseed oil and canola oil for Atlantic salmon and canola oil and palm oil for seabass

Dicentrarchus labrax respectively. The methods used in this trial are similar to those used in Wilson et al (2007), in that a single measurement of $\dot{M}O_{2\ rout}$ was taken prior to a measurement of physiological performance. Alternatively, Chatelier et al (2006) extrapolated back from oxygen measurements taken during swimming, whilst McKenzie et al (1997; 2000) took $\dot{M}O_{2\ rout}$ measurements every 10 min for 8 h. It is possible that the methods used in this thesis and Wagner et al. (2007) are not as robust in detecting the subtle changes in metabolic rate that would be expected with a trial such as the one conducted. That differences were found by McKenzie et al (1997; 2000) and not Wilson et al (2007) and Chatelier et al (2006) could lead one to believe that in order to truly determine if there is an effect on $\dot{M}O_{2\ rout}$ one must record metabolic rates over an extended period of time. Routine metabolic rates can significantly fluctuate over the course of a day, peaking when the fish are normally feeding and at the lowest when the fish is normally at rest. Furthermore, $\dot{M}O_{2\ rout}$ within any group of fish can be highly variable, with variability associated with differences in condition factor, growth rate and hierarchy within the group (Metcalfe et al., 1995; Cutts et al., 1998; Cutts et al., 2002). By taking a single measurement of $\dot{M}O_{2\ rout}$ there is a risk that the variability in $\dot{M}O_{2\ rout}$ within a given population of fish at a single time point may be greater than the variability in $\dot{M}O_{2\ rout}$ between the treatments, thus masking any effect that the treatment may have on $\dot{M}O_{2\ rout}$. In order to overcome this problem, multiple measurements of $\dot{M}O_{2\ rout}$ must be made over an extended time frame (McKenzie et al., 1997; 2000). Indeed it would be pertinent for a comparison to be made of the techniques employed in the aforementioned papers on the same population of fish in order to validate the

results. The results observed in Chapter 6 do suggest that analysing individual $MO_{2\ rout}$ and correlating the results with FA profiles is a much better approach than looking at group $MO_{2\ rout}$ and comparing it against group FA analysis.

7.5 Future directions for research

The results obtained in Chapter 4 indicated that there was a severe restriction on the swimming performance of Atlantic salmon affected by low levels of AGD when they were required to swim at or near maximum swimming capacity. However it is not known whether the reduction in swimming performance was due to a greater reduction in blood Po_2 values and hence a greater reliance on anaerobic metabolism, a blood chemistry permutation (i.e. change in blood pH and thus haemoglobin binding properties), a result of the cardiovascular dysfunction reported in other studies or a combination of a number of different factors (Powell et al., 2000; Powell and Nowak, 2003; Leef et al., 2005). Examining the cardiac output of a cannulated swimming fish with AGD whilst simultaneously taking blood chemistry measurements would answer some of the aforementioned questions, this approach has been used previously and allowed researchers assessing the impact of sea lice *Lepeophtheirus salmonis* on cannulated Atlantic salmon to ascertain the cause of the reduction in swimming performance, in that case a reduction in heart rate (Wagner et al., 2003). Furthermore, all research to date has examined the effect of AGD on the physiology of the fish under routine oxygen consumption conditions, therefore it is difficult to draw conclusions on the physiological impact of AGD on

Atlantic salmon under normal farm conditions, in which they are frequently exposed to periods of high intensity exercise (eg. freshwater bathing, tidal and cage movements). Previous research examining the pathophysiological effects of AGD in salmonids have demonstrated that there is no reduction in blood Po_2 values for Atlantic salmon with AGD under routine conditions, it is of particular interest to determine whether this statement holds true for salmon respiring at or near maximum metabolic rate (Powell et al., 2000; Powell and Nowak, 2003). Therefore it is recommended that, given the opportunity, future studies should incorporate techniques outlined in Leef et al (2005a; 2005b) and Powell et al (2002) for measuring cardiac output and blood gas parameters on actively swimming fish in order to determine whether the conclusions drawn from previous studies are relevant for actively swimming fish.

7.6 Conclusions

This study has highlighted the aimed to quantify the metabolic cost of gill diseases, specifically AGD and *Tenacibaculum maritimum* in Atlantic salmon. Furthermore, insight has been gained into some of the likely causes of the observed metabolic costs. As well as adding to the increasing amount of literature examining the effect of diet and disease on the physiology of fish, the results of this thesis have highlighted non-destructive methods that can potentially be used to quantify the impact of disease, disease treatment and nutritional status on the animal without the need for lethal sampling. The thesis also has shown that the nutritional status of the animal plays a minimal role in the host's physiological response to gill disease. In

conclusion, this thesis has added valuable insight into the metabolic changes associated with the complex interactions between nutrition, disease, metabolic rate and swimming performance.

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APPENDIX I

Table 1. Fatty acid content and lipid class composition of the whole carcass of Atlantic salmon smolt fed canola oil (CO), stearidonic rich oil (SO) diets and fish oil (FO) (g/100 g total fatty acids). P< 0.05 indicates significant difference between treatments.

FA content (mgg^{-1})	Intitial (±SE)	CO (±SE)	SO (±SE)	FO (±SE)	f	df	sig
14:0	3.7 ± 0.2b	1.7 ± 0.2a	1.7 ± 0.3a	6.0 ± 0.3c	61.5	3,30	0.00
16:0	15.2 ± 0.9a	11.9 ± 0.6a	13.2 ± 19a	22.4 ± 1.4b	12.9	3,30	0.00
18:0	4.3 ± 0.2	4.1 ± 0.2	5.6 ± 0.8	5.7 ± 0.4			
Other SFA	1.2 ± 0.1a,b	1.5 ± 0.2b	0.7 ± 0.1a	1.9 ± 0.2b	7.0	3,30	0.00
Total SFA	24.4 ± 1.4a	19.3 ± 1.1a	21.2 ± 3.0a	35.9 ± 2.1b	13.3	3,30	0.00
16:1w7c	6.7 ± 0.4b	3.2 ± 0.4a	3.0 ± 0.5a	11.0 ± 0.6c	68.5	3,30	0.00
18:1w9c	16.4 ± 1.6a	53.8 ± 1.7b	22.3 ± 3.2a	24.8 ± 1.7a	55.1	3,30	0.00
18:1w7c	3.7 ± 0.a,b	4.7 ± 0.2b,c	2.4 ± 0.4a	5.8 ± 0.4c	19.8	3,30	0.00
20:1w9c	1.4 ± 0.1a	2.3 ± 0.1b	1.4 ± 0.2a	1.7 ± 0.2a,b	4.9	3,30	0.00
Other MFA	1.5 ± 0.2	1.9 ± 0.1	1.2 ± 0.2	1.4 ± 0.2			
Total MFA	29.7 ± 2.4a	65.8 ± 2.2c	30.3 ± 4.4a	44.7 ± 2.5b	29.3	3,30	0.00
18:3w3 ALA	0.7 ± 0.2a	2.8 ± 0.2a	13.4 ± 1.9b	1.3 ± 0.2a	36.7	3,30	0.00
18:4w3 SDA	1.7 ± 0.2a	2.0 ± 0.1a	9.8 ± 1.5b	2.8 ± 0.2a	22.9	3,30	0.00
20:4w3	0.9 ± 0.1a,b	0.5 ± 0.1a	1.6 ± 0.2c	1.2 ± 0.2b,c	8.7	3,30	0.00
20:5w3 EPA	5.4 ± 0.3b	2.3 ± 0.2a	2.7 ± 0.5a	9.0 ± 0.5c	57.8	3,30	0.00
22:5w3 DPA	2.2 ± 0.1b	1.0 ± 0.1a	1.1 ± 0.2a	2.9 ± 0.4b	13.1	3,30	0.00
22:6w3 DHA	9.3 ± 0.4b	1.6 ± 0.8a	1.4 ± 0.9a	12.3 ± 1.0b	42.9	3,30	0.00
Other w3	0.4 ± 0.0b	0.1 ± 0.0a	0.1 ± 0.0a	0.4 ± 0.1b	11.3	3,30	0.00

Total w3	20.6 ± 1.1b	10.3 ± 1.0a	30.2 ± 4.8b	30.0 ± 2.2b	11.1	3,30	0.00
18:2w6	4.3 ± 0.5a	16.3 ± 0.6b	15.8 ± 2.2b	7.6 ± 0.5a	21.0	3,30	0.00
18:3w6	0.2 ± 0.1a	1.3 ± 0.1a	5.5 ± 0.8b	0.3 ± 0.1a	33.6	3,30	0.00
20:3w6	0.2 ± 0.1a	1.1 ± 0.0b	1.6 ± 0.2c	0.3 ± 0.0a	32.6	3,30	0.00
20:4w6	0.7 ± 0.0	0.9 ± 0.1	0.6 ± 0.1	0.9 ± 0.1			
22:5w6	0.2 ± 0.0a	2.8 ± 0.9b	2.8 ± 0.8b	0.1 ± 0.0a	6.6	3,30	0.00
Other w6	0.1 ± 0.0a,b	0.0 ± 0.0a	0.0 ± 0.0a	0.2 ± 0.0b	5.2	3,30	0.01
Total w6	5.8 ± 0.6a	22.4 ± 1.2b	26.4 ± 3.4b	9.4 ± 0.6a	24.9	3,30	0.00
Other PUFA	1.3 ± 0.1b	0.6 ± 0.1a	0.5 ± 0.1a	2.0 ± 0.3b	17.2	3,30	0.00
Total PUFA	27.7 ± 1.8a	33.3 ± 1.6a	57.2 ± 8.3b	41.4 ± 2.9a,b	6.9	3,30	0.00
w3/w6	3.8 ± 0.4b	0.5 ± 0.1a	1.1 ± 0.1a	3.2 ± 0.2b	78.9	3,30	0.00
Lipid Class (mgg^{-1})							
TAG	77.5 ± 7.3a	107.8 ± 8.9b	103.0 ± 9.8a,b	100.5 ± 9.9b	3.1	3,30	0.04
FFA	0.9 ± 0.2	0.2 ± 0.1	0.9 ± 0.6	0.2 ± 0.0			
ST	1.2 ± 0.1b	0.6 ± 0.1a	0.8 ± 0.1a,b	0.6 ± 0.1a	6.5	3,30	0.02
PL	7.8 ± 0.7	7.9 ± 0.9	7.7 ± 0.9	9.2 ± 0.9			
Lipid content (mgg^{-1})							
Wet	81.8 ± 5.1a	118.4 ± 4.8a,b	108.7 ± 15.8a,b	122.0 ± 6.4b	3.2	3,30	0.04
Dry	264.9 ± 15.5a	317.5 ± 12.8a,b	318.1 ± 12.4a,b	347.8 ± 19.1b	4.4	3,30	0.01

SO, stearidonic oil diet; CO, canola oil diet; FO, fish oil diet; SFA, Saturated fatty acids; MUFA, Monounsaturated fatty acids; PUFA, Polyunsaturated fatty acids; DHA, Docosahexaenoic acid; DPA, Docosapentaenoic acid; EPA, Eicosapentaenoic acid; ETA, Eicosatetraenoic acid; SDA, Stearidonic acid; LA, Linoleic acid; ALA, α-Linolenic acid; ; LA, Linolenic acid: GLA, γ-Linolenic acid, AA, Arachidonic acid; TAG; Triacylglycerol; FFA, Free fatty acid; ST, Sterol; PL, Polar lipid; WW, Wet weight; *f*, Mean sum of squares.

[a,b,c,d] Mean values across the row not sharing a common superscript were significantly different as determined by Tukey-Kramer HSD; *df*=3,30. $P<0.01$ * indicates $P<0.05$

[1] Includes 15:0, 17:0, 20:0, 22:0 and 24:0

[2] Includes 16:1ω9, 16:1ω5, 18:1ω5, 20:1ω7, 22:1ω9, 22:1ω11 and 24:1ω9

[3] Includes 21:5ω3 and 24:6ω3

[4] Includes 20:2ω6, 22:4ω6 and 24:5ω6

[5] Includes 16:2ω4, 16:3ω4 and 18:2ω9

[6] Determined gravimetrically and confirmed by TIC-F

Table 2 Fatty acid content and lipid class composition of the white muscle samples of Atlantic salmon smolt fed canola oil (CO), stearidonic rich oil (SO) diets and fish oil (FO) (g/100 g total fatty acids). $P < 0.05$ indicates significant difference between treatments.

FA content (mgg^{-1})	Intitial (±SE)	CO (±SE)	SO (±SE)	FO (±SE)	f	df	sig
14:0	0.7 ± 0.1a,b	0.3 ± 0.1a	0.3 ± 0.1a	1.3 ± 0.3b	48.2	3,40	0.00
16:0	3.5 ± 0.4	3.0 ± 0.4	3.7 ± 0.6	4.8 ± 0.8			
18:0	0.9 ± 0.1	1.0 ± 0.2	1.5 ± 0.3	1.1 ± 0.2			
Other SFA	0.3 ± 0.0	0.3 ± 0.1	0.1 ± 0.0	0.4 ± 0.1			
Total SFA	5.4 ± 0.6	4.7 ± 0.7	5.6 ± 1.0	7.7 ± 1.4			
16:1w7c	1.2 ± 0.2a,b	0.5 ± 0.1a	0.4 ± 0.1a	2.1 ± 0.5b	8.0	3,40	0.00
18:1w9c	3.4 ± 0.9a	12.8 ± 2.3b	5.3 ± 1.3a	4.5 ± 0.9a	8.0	3,40	0.00
18:1w7c	0.8 ± 0.1a,b	1.0 ± 0.1a,b	0.5 ± 0.1a	1.2 ± 0.2b	3.9	3,40	0.02
20:1w9c	0.3 ± 0.0	0.5 ± 0.1	0.3 ± 0.1	0.4 ± 0.1			
Other MFA	0.2 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1			
Total MFA	5.9 ± 1.0a	15.2 ± 2.6b	6.9 ± 1.6a	8.5 ± 1.7a,b	4.6	3,40	0.01
18:3w3 ALA	0.0 ± 0.0a	0.8 ± 0.1a	4.4 ± 1.0b	0.3 ± 0.1a	14.5	3,40	0.00
18:4w3 SDA	0.3 ± 0.0a	0.5 ± 0.1a	3.1 ± 0.7b	0.6 ± 0.1a	12.1	3,40	0.00
20:4w3	0.2 ± 0.0a	0.1 ± 0.0a	0.5 ± 0.1b	0.3 ± 0.1a,b	5.7	3,40	0.00
20:5w3 EPA	1.3 ± 0.2a,b	0.6 ± 0.1a	0.7 ± 0.1a	2.2 ± 0.4b	9.4	3,40	0.00
22:5w3 DPA	0.4 ± 0.1a,b	0.2 ± 0.0a	0.2 ± 0.0a	0.7 ± 0.1b	8.5	3,40	0.00
22:6w3 DHA	2.9 ± 0.3b	1.4 ± 0.1a	1.4 ± 0.2a	3.9 ± 0.5b	15.5	3,40	0.00
Other w3	0.1 ± 0.0a,b	0.0 ± 0.0a	0.0 ± 0.0a	0.1 ± 0.0b	7.0	3,40	0.00
Total w3	5.2 ± 0.6a,b	3.6 ± 0.3a	10.4 ± 2.1b	8.1 ± 1.3a,b	4.9	3,40	0.01

18:2w6	0.9 ± 0.2a	3.3 ± 0.5a,b	4.6 ± 1.0b	1.5 ± 0.3a	6.1	3,40	0.00
18:3w6	0.0 ± 0.0a	0.4 ± 0.1a	1.7 ± 0.4b	0.1 ± 0.0a	13.5	3,40	0.00
20:3w6	0.0 ± 0.0a	0.4 ± 0.1b,c	0.6 ± 0.1c	0.1 ± 0.0a,b	11.7	3,40	0.00
20:4w6	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0			
22:5w6	0.0 ± 0.0a,b	0.0 ± 0.0a	0.0 ± 0.0a,b	0.1 ± 0.0b	4.9	3,40	0.01
Other w6	0.0 ± 0.0a	0.0 ± 0.0a,b	0.0 ± 0.0a,b	0.1 ± 0.0b	3.5	3,40	0.03
Total w6	1.2 ± 0.2a	4.3 ± 0.5a,b	7.2 ± 1.6b	2.0 ± 0.5a	7.6	3,40	0.00
Other PUFA	0.3 ± 0.0a,b	0.1 ± 0.0a	0.1 ± 0.0a	0.5 ± 0.1b	8.1	3,40	0.00
Total PUFA	6.6 ± 0.8a	8.0 ± 0.5a	17.7 ± 3.6b	10.6 ± 1.9a,b	4.3	3,40	0.01
w3/w6	4.8 ± 0.4b	1.1 ± 0.3a	1.5 ± 0.0a	4.7 ± 0.4b	53.1	3,40	0.00
Lipid Class (mgg^{-1})							
TAG	11.7 ± 4.6	20.0 ± 5.9	17.7 ± 6.0	20.3 ± 5.0			
FFA	0.7 ± 0.2b	0.1 ± 0.0a	0.1 ± 0.0a	0.1 ± 0.0a	15.9	3,40	0.00
ST	0.2 ± 0.0	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.0			
PL	5.3 ± 1.3	5.4 ± 0.6	7.2 ± 1.0	6.3 ± 0.5			
Lipid content (mgg^{-1})							
Wet	17.9 ± 2.9	28.0 ± 3.4	30.1 ± 6.2	26.8 ± 4.9			
Dry	80.4 ± 12.9	109.8 ± 9.7	108.3 ± 20.4	108.0 ± 17.4			

df=3,40. $P<0.01$ * indicates $P<0.05$
Abbreviations and other footnote definitions, see Table 1 Appendix I.